The Functional Significance of Type IV Pilus Proteins for Competence and Effects of Environmental Conditions on Competence in *Acinetobacter baylyi*

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By

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Abstract.

Acinetobacter baylyi are naturally competent soil bacteria. Natural transformation is the acquisition of new genetic material via the uptake of foreign, exogenous DNA. Competence is the physiological state some bacterial species may realize in order for natural transformation to occur. Natural transformation, and therefore competence, is clinically relevant, as natural transformation serves as a chief method by which antibiotic-resistant genes are dispersed amongst the bacterial population. *A. baylyi* serves as an ideal model organism to model natural transformation. *A. baylyi* are easy to cultivate *in vitro*, may be genetically modified with ease, and there is a complete library of single gene deletion mutants available for research use.

Our first goal was to test the role of Type IV pilus (T4P) proteins in competence using a novel surface-associated quantitative protocol. From the French collection, we obtained knockout mutants lacking proteins predicted to be important for comprising a T4P or for uptake of DNA across the inner membrane. Transformation of cells on a nutritive agar surface allowed for quantitative determination of transformation efficiency over nine orders of magnitude. Using this method we determined which genes were necessary for competence.

Under the conditions we tested, genes absolutely required for transformation in *A. baylyi* include genes encoding the basal apparatus of a T4P (*comM*, *pilF*, *pilC*, *pilU*, and *pilT*), the gene encoding the inner membrane DNA translocation protein (*comA*), the gene encoding the major pilin (*comP*), genes encoding minor pilins (*pilV, fimT*), and the gene encoding the pilus tip protein (*comC*).

Mutations in genes encoding for a periplasmic protein that helps target DNA to the *comA* channel (*comEA*), conserved hypothetical protein (*CHP*), genes encoding for a signal transduction response and regulatory receiver (*pilG*, *pilR*, and *pilS*), and a gene encoding for a minor pilins, *comF* and *comE*, resulted in a 2-4 log loss in competence. Using transformation on a surface instead of in liquid, we have discovered that a T4P, including its major pilin, is required for transformation *A. baylyi*.

Further, in order to determine conditions under which *A. baylyi* are most competent in LB media, a simple broth comprised of three ingredients in an easy ratio, we tested additives to LB broth. Though *A. baylyi* may be grown in a variety of different medias, our laboratory chooses to grow ADP1 cells in LB because of its ubiquity in bacterial labs, low cost, and high rates of natural transformation.

Our experiments address whether we may increase the efficacy of LB by altering growing temperature or infusing it with a variety of experimental additives. Overall, we found that *A. baylyi* cells are competent in nearly all LB conditions tested, but notably, rates of transformation slightly increase in LB+succinate but drastically decrease in LBK+Fe-deficient.

Keywords: *Acinetobacter baylyi*, competence, transformation, Type IV pili (T4P)

Introduction.

Horizontal gene transfer:

The basis of evolution is genetic variation, which can be achieved by mutation or genetic intermingling. Bacteria, which reproduce by binary fission, require non-meiotic mechanisms to achieve genetic intermingling and to evolve. These mechanisms are mutation and more saliently, horizontal gene transfer (HGT), which can provide bacteria an evolutionary advantage, as transferrable genes include those conferring antibiotic resistance and virulence factors (Davies, 2010). HGT comprises three separate processes: conjugation, transduction, and natural transformation. Conjugation, the most common form of HGT, is the transfer of plasmid DNA through a created physical connection between two bacterial cells, a mating bridge (Salyers and Whitt, 2001). Transduction involves a transfer of DNA from one bacterial cell to another via an auxiliary virus. Genes from a host bacterium are incorporated into the genome of a bacteriophage then carried to and subsequently integrated into the genome of another host cell when the bacteriophage initiates another cycle of infection (Salyers and Whitt, 2001).

Finally, natural transformation is the process of HGT in which exogenous DNA is introduced to bacteria, transported across the cell envelope, and subsequently incorporated into the genome. Yet of the three, natural transformation is the most versatile and provides the broadest gene transmission (Herzberg *et al*, 2000).

Natural transformation and competence:

Of the three HGT processes, natural transformation is the least understood, likely because only a small fraction of bacterial species are competent, that is, able to realize the

regulated physiological state necessary for natural transformation to take place (Mell and Redfield, 2014). Transformation can also be induced artificially *in vitro*, as with chemically induced or electrically shocked bacteria (Tu, 2008). Techniques such as electroporation create pores to increase permeability in the bacterial membrane through which genetic material may enter. For example, artificially induced competence in *Escherichia coli* makes for an efficient and convenient way for simplified molecular cloning methods in both biotechnology and research laboratories.

However, genera such as *Streptococcus*, *Bacillus*, *Acinetobacter*, *Neisseria*, and *Pseudomonas* are naturally competent (Tu, 2008). Our study utilizes *Acinetobacter baylyi*, ADP1, as a model organism in which to study natural competence (see ***Acinetobacter baylyi*, ADP1, as a model organism**). It is impressive that such a broad range of bacteria genera possess this conserved ability to import foreign DNA, indicating that competence must be some evolutionary survival tactic. Frederick Griffith's 1928 research with two strains of *Streptococcus pneumoniae* demonstrated that a combination of heat-killed virulent smooth strain with nonvirulent rough strain could still be lethal to mice. It was understood that the live rough strain acquired its virulence factors from the free-floating genetic material of the lysed virulent smooth strain. Similarly, the exogenous DNA taken up by naturally competent bacteria *in vivo* are leftovers from deceased organisms, such as plant matter.

Oswald Avery, Colin MacLeod, and Maclyn McCarty's 1944 research further proved that this "transforming principle" was, in fact, DNA and not protein. Further, natural transformation is understood to have aided in the evolution of single-celled eukaryotic organisms from Archaea and prokaryotic ancestors (Averhoff, 2004). Specific

environmental conditions such as access to nutrients or neighboring cell density are thought to induce temporary competence in some species (Thomas and Nielsen, 2005). Natural transformation involves 20-50 proteins, which work together to create a surface protein machine; the details of such machinery are relatively unknown (Thomas and Nielsen, 2005; Chen and Dubnau, 2004; see Figures 1 and 2).

During natural transformation, bacteria uptake exogenous, foreign DNA and subsequently incorporate it into their own genome. Transformation begins with an early competence signaling pathway, though the precise environmental conditions that induce competence in ADP1 are not fully understood. Signaling processes compose a pathway of both intra- and extracellular signals the lead to the induction of competence (Palmen and Hellingwerf, 1997). Interestingly, many of the major proteins involved in this sensory pathway are conserved across competent organisms and their species-specific pseudopilus complexes (Palmen and Hellingwerf, 1997).

When the sensory pathway is triggered, bacteria must assemble the structural components of the competence machinery, which must serve four purposes: recognizing the potential DNA, binding to the DNA, processing DNA for importation, and importing the DNA into the cell interior. Once within the cell interior, the foreign DNA may be used in several ways: first, to serve as a template in DNA repair, second, as a source of carbon, nitrogen, and phosphorous, and third, to acquire new, fitness-enhancing traits such as genes conferring antibiotic resistance.

Competence is understood to occur via a “competence nanomachine,” some mechanical apparatus that serves to collect exogenous DNA and allow for its import across the periplasm. Construction of such a machine necessitates the expression of genes

for specific proteins that make up said machine. Yet, the minutiae of the uptake machinery are not totally understood in any system (Chen and Dubnau, 2004). Compellingly, it is thought that it takes longer than a single bacterial generation to construct the competence nanomachine. Bacterial flagella are different from competence nanomachines but are of similar complexity. Flagella are lengthened outside of the cell and the rate of flagellum growth does not change as it lengthens (Evans *et al*, 2013; Turner, 2012). Zimmer (2009) writes that for *E. coli*, the creation of a flagellum can take a couple of hours, several generations of life. The energy expenditure for a bacterial cell to commit to building a flagellum or competence nanomachine is significant – beginning with transcription, it takes roughly 6 seconds to make a protein. Competence pili are comprised of about 50 proteins in the basal body. 1500 pilins are extended or retracted per second (Burrows, 2005). Therefore, it takes roughly 2.5 hours for create all the necessary protein components to extrude a single pilus for a single second, the time equivalent for five new generations. Thus, for a cell to expend such a vast quantity of energy on the physiological process, competence is indeed of incredible importance for species that may achieve this regulated state.

Evidently, the import of exogenous DNA entails several distinct phases. *Acinetobacter baylyi*, our model organism (see ***Acinetobacter baylyi*, ADP1, as a model organism**) is a Gram-negative bacterium that requires foreign DNA to be transported across the outer membrane, the cell wall and periplasm, and finally, the cytoplasmic membrane. In an ATP-required process, DNA is transported across the outer membrane via secretin complexes, through the cell wall via a pilus-mediated transport, and lastly,

DNA is moved across the cytoplasmic membrane via a polytopic membrane protein (Chen and Dubnau, 2004; Figure 3).

Yet, further complicating matters, *A. baylyi* can bind to all forms of substrate double-stranded DNA (Palmen and Hellingwerf, 1997). Likewise, this process is not completely understood, as in Gram-negative bacteria, double-stranded DNA enters the cell wall, but somehow, one strand is degraded and only single-stranded DNA is permitted into the cytoplasmic membrane. A theoretical model of the competence pseudopilus used for DNA import includes other proteins homologous to others in T4P and T2SS. Finally, through a RecA-dependent recombination mechanism in which the Rec-A protein binds to the incoming substrate DNA strand and the corresponding sequence within the host genome, the single strand of DNA is incorporated into the circular bacterial chromosome (Porstendörfer *et al*, 1997).

Antibiotic resistance:

Natural transformation is a burgeoning field for intensive research and clinical applications. Antibiotic resistant bacteria are an increasingly serious health problem and HGT, particularly natural transformation, facilitates. Rapid reproduction combined with environmental hardiness allow for dispersion of resilient genes and extensive resistance across and between bacterial species, especially competent ones. Some species including *Haemophilus influenza* and *Neisseria gonorrhoeae* require certain sequences present in DNA in order for that DNA to be imported (Chen and Dubnau, 2004). Yet *Bacillus subtillus*, *Streptococcus pneumoniae*, and *Acinetobacter baylyi* do not have any requirement for specific DNA uptake sequences (Palmen and Hellingwerf, 1997; Chen

and Dubnau, 2004; Lang and Lostroh, 2013). Therefore, it is possible for gainful genetic information to be transferred amongst species or even within species amongst individuals. Pontiroli *et al* (2009) confirmed *A. baylyi*'s high rate of natural transformation amongst species, as transformed bacterial cells expressed both antibiotic resistance and green pigmentation from transgenic tobacco plants.

Most saliently, examples of clinically relevant, increasingly resistant to antibiotics, and competent bacteria include *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, and *Acinetobacter baumannii* (Slager *et al*, 2014; Tapsall, 2005; Maragakis and Perl, 2008). Elaborating on the final example, *A. baumannii* are widespread in hospital settings and have a high propensity to cause extensive, often multifacility, nosocomial outbreaks (Fournier *et al*, 2006). As a result of being competent, *A. baumannii* are conspicuously resistant to many available antibiotics, including penicillins, cephalosporins, fluroquinolones, and aminoglycosides (Manchanda *et al*, 2010). A high rate of natural transformation combined with ability to attach to both biotic and abiotic surfaces make these bacteria an increasingly dangerous pathogen (Alyamani *et al,* 2014). As illustrated by *A. baumannii*, antibiotic resistance is an increasing human health problem that is accelerated by competent bacteria, thus underscoring the importance of research on competence.

***Acinetobacter baylyi*, ADP1, as a model organism:**

In order to study competence, we use *Acinetobacter baylyi*, formerly known as *A. calcoaceticus*, a competent but less pathogenic cousin of *A. baumannii* as an advantageous model organism (Vaneechoutte *et al*, 2006). DNA uptake by *A. baylyi in*

vitro is swift – 60 base pairs per second (Palmen and Hellingwerf, 1997). Palmen and Hellingwerf (1997) estimate that ~0.1% of large internalized foreign DNA fragments are successfully incorporated into the *A. baylyi* genome through homologous recombination. There is no data disclosing the rate of recombination for short linear DNA.

A. baylyi is easy to culture *in vitro* and has a relatively small genome of 3.6 Mb, making genetic manipulation straightforward (de Berardinis *et al*, 2008). In addition, its entire genome is sequenced, encoding 3325 predicted coding sequences (Barbe *et al*, 2004) and a full library of single-gene deletion mutants of ADP1 is readily accessible for research use (de Berardinis *et al*, 2008). Of the 3,197 annotated protein-encoding genes in ADP1, 97% have been assigned an essential/dispensable status (de Berardinis *et al*, 2008). Our laboratory obtained all mutant strains of *A. baylyi* from this French collection. Further, *A. baylyi* is competent at varying levels during all phases of its life cycle. However, competence maximized after transfer of overnight incubation into fresh medium (Lang and Lostroh, 2013). A final and compelling reason for using *A. baylyi* as a model is its own emerging pathogenicity, thus demonstrating its similarity to *A. baumannii* and other competent pathogens. *A. baylyi* was until recently considered non-pathogenic, but through natural transformation it has acquired virulence factors and infectious traits, and has thereby become a human disease agent causing nosocomial infections in the immunocompromised (Chen *et al*, 2008). Its emergent pathogenicity accentuates the utility of *A. baylyi* as a model competent bacterium; however, *A. baylyi* are still classified as Biosafety Level 1 and research with these organisms may be performed on standard open-air laboratory benches, rendering it an accessible model organism.

Type IV pili and structure:

As mentioned previously, natural transformation is the least understood of the three HGT processes. The competence pilus is understood to be similar in structure to Type IV pili (T4P) and Type II secretion systems (T2SS). The conserved proteins for all three systems include a cytoplasmic adenoside triphosphatase (ATPase) of the AAA^+ ATPase superfamily, a polytopic membrane protein, a pre-pilin peptidase, and several pilins or pilin-like proteins (Chen *et al*, 2005). Averhoff (2004) proposes that the T4P and T2SS are composed of homologous, or even identical, structural proteins. It is important to recognize that T4P are homologous to T2SS and *not* to Type IV secretion systems, which are instead homologous to conjugation pili. However, in this study, we will focus on T4P.

T4P are long, fibrous structures external to the cell. Research with *Pseudomonas aeruginosa* has demonstrated T4P to be involved in twitching motility, which is a mobility pattern of single-celled organisms to move across surfaces, host cell adherence, biofilm formation, bacteriophage infection, and finally, natural transformation (Schaik *et al*, 2005). Recently, it was found that T4P on pathogens provoke the host immune response and as such, are potential drug and vaccine targets (Craig *et al*, 2004). Further, researchers observed that *P. aeruginosa* T4P bound DNA without sequence specificity, similar to *A. baylyi*'s lack of necessity for such (Schaik *et al*, 2005). Gram-negative bacteria utilize T2SS to translocate proteins from the periplasm across the outer membrane (Korotkov *et al*, 2012). Generally, secretion systems mediate the transfer of DNA or protein substrates.

Members of the *Acinetobacter* genus have two structurally different appendages, thin and thick pili as detected by examining stained specimens using electron microscopy (Gohl *et al*, 2006). *A. baylyi* is no exception to its genus, as electron micrographs suggest that bundled thin pili have a diameter of 3 nm and singular thick pili have a diameter of 6 nm (Link *et al*, 1998). These dimensional estimates are given by stained appendages and it is important to note that electron micrograph staining might alter dimensions. Thin pili are thought to be associated with adhesion to hydrophobic surfaces, while thick pili are thought to play a role in twitching motility (Gohl *et al*, 2006). The Averhoff laboratory purified proteins comprising the *A. baylyi* thin pilus and cloned the gene encoding its major pilin, *acuA* (Gohl *et al*, 2006). A mutation of *acuA* led to a loss of thin pili and though mutant bacteria were unable to adhere to biotic and abiotic surfaces, the bacteria were still competent. Thus, thin pili function in adhesion, but not competence or twitching motility. Researchers were unable to deduce protein components of thick pili, but it was known that they are polar and play some role in twitching motility.

T4P are typically 5-7 nm in diameter and extend roughly 3 μm in length (Mattick, 2002). A central group of 12-15 proteins the bacterium produces serve to aid in assembly and function (Jarrell and McBride, 2008). Through evaluation of pili structure in *N. gonorrhoeae* and *P. aeruginosa*, a model of T4P was developed: the structure inserts into the cytoplasmic membrane and spans the periplasmic space and outer membrane via a helical arrangement of five subunits per turn (Herzberg *et al*, 2000). Burrows (2005) suggests there are 500-1000+ pilin subunits per pilus with three pilins per turn; a 30 angstrom gain in length per turn. Synthesizing a pilus is certainly a time and energy commitment – the time required to construct a single pilus exceeds that of a single

bacterial generation, without accounting for the synthesis of associated processing and export machinery.

Using *P. aeruginosa* as a model, Jarrell and McBride (2008) found that the pilus fiber is chiefly composed of a subunit pilin protein, PilA. Protein PilB is necessary for pilus extension and PilT is involved in retraction (Jarrell and McBride, 2008). *A. baylyi*, our model organism, has two PilT homologs: PilT and PilU. Additionally, it has one PilB homolog, also called PilB. In all Gram-negative bacteria, including *A. baylyi*, secretin ComQ, sometimes known as PilQ, creates a pore in the outer membrane through which the pilus passes. *A. baylyi* has two secretins, encoded by ACIAD0294 and ACIAD3355, the latter is called ComQ. ACIAD0294 is grouped on the chromosome near Type II secretion system genes so it is likely used for secretion, not transformation.

Though we have information on the proteins required for T4P biogenesis, the actual pili synthesis process of unknown. Though the central pilus fiber is composed of Type IV pilin, minor pilins are also involved in the synthesis (Chen and Dubnau, 2004). Somehow, the pilus filament extends beyond the cell membrane. Burrows (2012) suggests that the pilus might grow from the proximal end instead of the tip, as flagella does. Based on x-ray crystallography, the Averhoff laboratory has constructed a hypothesized pilus fiber model (Averhoff, 2004; see Figure 1).

Type IV pili and competence:

Chen *et al* (2005) found that *N. gonorrhoeae* produces T4P and determined many proteins required for pilus formation are also required for DNA uptake and transformation. There is correlation between competence ability and the presence of T4P

in bacteria. The competence nanomachine spans the width of the cell envelope and is united to a DNA translocation complex at the cell membrane on the cytoplasmic side (Chen *et al*, 2005). Yet, because available diagrams of competence structures are purely speculative, whether natural transformation is even mediated by a pilus is unknown. Chen *et al*'s (2005) pilus model supports that the pilus is not involved in direct transformation, as the cavity in the middle of the pilus is narrow and hydrophobic, two traits that are hostile for the passage of DNA. Hence, Chen *et al* (2005) refer to such as a competence "pseudopilus." This competence pesudopilus is similar in structure to T4P, utilizing the same genes in order to be built as a T4P, but instead acts to carry exogenous DNA to the transport machinery during natural transformation (Chen *et al*, 2005).

Initially, dsDNA must interact with the competence cell's surface, however, this process is not completely understood. In *B. subtilis*, a Gram-positive model, ComEA, a membrane-bound dsDNA binding protein is required for transformation, but its responsibility in residual binding remains unidentified (Chen *et al*, 2005). However, in Gram-negative bacteria, such as our model organism, *A. baylyi*, a single strand of DNA passes through the cytoplasmic membrane while its complement is degraded. DNA is transported into the cytosolic space in a linear fashion and a required free end is understood to initiate the transport process (Chen *et al*, 2005).

DNA likely enters the periplasm through the secretin channel; secretins are proteins involved in the extrusion of T4P and aid in crossing the outer membrane. They form stable, donut-like multimeters in the outer membrane with an aqueous central cavity. This aqueous central cavity is 6.5 nm in diameter, large enough to accommodate the passage of dsDNA (typically 2.4 nm in diameter), supporting the idea that DNA likely

enters the periplasm via the secretin channel (Chen *et al*, 2005). The most similar ADP1 protein to the competence secretin in *N. gonorrhea* is ComQ, but diameters of the ComQ channel likely vary depending on species. Indeed, prior research has demonstrated that *comQ* ADP1 knockouts are not competent (Lieber, 2014).

Type IV pili relative to twitching motility and *Pseudomonas aeruginosa*:

Though this thesis emphasizes the importance of the T4P during competence, it is necessary to acknowledge T4P's role in twitching motility, a form of jerky bacterial movement across a moist surface. Flagella are not used during twitching. Through the extension, tethering, and then retraction of polar T4P, twitching is not only important in bacterial locomotion, but plays a significant role in colonization of surfaces when nutrients are abundance and/or to aggregate cells in biofilm and fruiting body formation in *Myxococcus* bacteria (Mattick, 2002). In fact, twitching motility was first discovered through studies of *A. baylyi* (Mattick, 2002).

Currently, *P. aeruginosa* is the chief model organism with which twitching is studied. Though *P. aeruginosa* produces T4P, it does not undergo natural transformation; however, its cousin, *P. stutzeri*, is competent. In fact, research from the Schaik *et al* laboratory suggests that *P. aeruginosa* T4P can in fact function in DNA uptake, as the PilA gene can restore transformation in a *P. stutzeri pilA* mutant (2005). Strikingly, the T4P major pilin, PilA, from *P. aeruginosa* complements a *pilA* null mutation in naturally competent *P. stutzeri,* indicating that T4P have a conserved role during competence. Though the T4P of *P. aeruginosa* do not perform natural transformation, Schaik *et al* (2005) hypothesizes that the role of T4P in transformation is to increase DNA uptake.

The pili bound both eukaryotic and prokaryotic DNA in a nonspecific fashion, increasing the amount of available exogenous DNA and therefore increased DNA uptake (Schaik *et al*, 2005).

Research question:

It is understood that T4P protein homologues are involved in transformation ability of many bacteria. Further, the similarities between proteins involved in DNA uptake and T4P systems suggest that all belong to a family of evolutionary related systems containing cell envelope spanning structures with conserved components (Porstendörfer *et al*, 1997). Our lab asks many questions: Are ADP1 pili involved in natural transformation? Are the T4P in *A. baylyi* involved in immediate DNA uptake and binding or perhaps, do the pilin-like components of the transformation system create a nanomachine serving a different purpose? In the first part of our experiment, ***The Functional Significance of Type IV Pilus Proteins for Competence***, we specifically ask: Are certain knockout mutants (see comprehensive list in Table 1) competent? Thus, which proteins of the competence pilus are absolutely necessary for competence to occur? Still, the precise structure of the *A. baylyi* competence machine remains unknown.

Leah Lieber, a former research student in the Lostroh laboratory investigated such T4P proteins and their relationship in competence, twitching motility, both, or neither. We hypothesize that mutants lacking genes necessary for DNA uptake will not be competent. Similarly, mutants lacking major genes encoding for major pilin proteins will not be competent. We suspect that genes necessary to create minor pilin proteins might result in reduced competence, but not total loss. In a continuation of Leah Lieber's

research evaluating functional significance of T4P proteins in *A. baylyi*, we selected a collection of genes based on sequence homology to proteins in *N. gonorrhoeae* and *P. aeruginosa* relative to competence. Specifically, we measured mutant transformation versus wild type transformation in order to draw conclusions to the relative function and importance of the missing gene and finally, construct a proposed diagram of an *A. baylyi* competence machine (see Figure 4). Because it is thought that competence and twitching are intrinsically linked, we also (see Figure 5). Ultimately, we found that genes *comM*, *pilF*, *pilC*, *pilU*, *pilT*, *comA*, *comP*, *pilV*, *fimT*, *comC* are necessary for competence, while genes *comEA*, *CHP*, *pilG*, *pilR*, *pilS*, *comF*, and *comE* result in defects in competence, but are not wholly necessary.

Further, to facilitate its use as a model organism, in a separate experiment ***Effects of Environmental Conditions on Competence in* Acinetobacter baylyi**, we continue with a study comparing *in vitro* competence conditions for *A. baylyi* grown in the ubiquitous and inexpensive medium Luria Bertani broth (LB). The Lang and Lostroh lab of the Colorado College in Colorado Springs, CO seeks to better understand the physical structure of *A. baylyi*'s transformation machinery. Our laboratory has developed a protocol in which we may image ADP1 cells with atomic force microscopy (AFM) that are producing "appendages," pili that may be related to competence, twitching motility, or other physiological processes that may involve pili. The latter half of my experimentation sought to determine the ideal LB broth composition in which competence for viewing using AFM is maximized.

Most previous studies of ADP1 transformation efficiency were done in liquid *Acinetobacter* minimal media (Averhoff *et al*, 2008; Juni, 1974). However, *Acinetobacter*

minimal media is complex, expensive, and time-consuming to make. It requires extensive preparation of numerous chemical compounds, adding room for human error. Further, the chemicals required are costly – costing approximately twice as much as general purpose LB. Finally, minimal media is time consuming to prepare – it takes researchers in this project three times longer to prepare than general purpose LB. Given these factors, our lab and several other ADP1 researchers use LB media for studying competence in *A. baylyi*. LB is commonly known to have transformation rates similar to minimal media, but in contrast to minimal media, LB is a ubiquitous media in bacterial labs, cost-effective, and has only three ingredients. Considered an all-purpose media, LB is comprised of 1% tryptone, 0.5% yeast extract, and 1% table salt, all of which are easily obtainable, inexpensive, and easily mixed in a 2:1:2 ratio.

While LB alone is a useful media for ADP1 transformation, there are a number of supplements easily added to its recipe or temperature modification, which have the potential to positively affect competence. Here we test these supplements to find an optimum formulation of LB based media for studying competence in the model organism *A. baylyi*. We tested liquid experimental environmental conditions including replacing sodium salts with potassium salts, adding succinate, removing iron, and adding divalent cations. We hypothesized that LB infused with a component known to upregulate cellular metabolism might heighten transformation efficiency and thus, maximize competence. Further, here we measure competence of *A. baylyi* in LB as a function of incubation temperature to ascertain if there is an optimum temperature. Knowledge of these conditions is an important starting point for researchers using *A. baylyi* as a model organism to study competence. Most notably, our results found that ADP1 cells are

competent across all tested environmental conditions, but slightly more competent in LB+succinate and severely less competent in LBK+Fe-deficient.

Methods.

Bacteria and media:

We used *A. baylyi* strain ATCC33305/ADP1/BD413 as a wild type. Mutants and their respective ACIAD numbers used in this study are listed **in Table 1.** We obtained this set of tdk-kan knockout mutants from the French collection of knockouts (de Berardinis *et al*, 2008). For a more comprehensive breakdown of all recipes, please see Appendix 1. We streaked wild type ADP1 on minimal media plates with recipe: 25 mL 0.5 M KH_2PO_4, 10 mL 10% $(NH_4)_2SO_4$, 1 mL concentrated base, 3.35 g Na_2HPO_4, 18 g BD Bacto™ Agar, 10 mL of 1 M succinate, and 754 mL dH_2O. To make concentrated base, we dissolved 20 g nitrolotriacetic acid and 14.6 g KOH in 600 mL of dH_2O. We then added 28.9 g anhydrous $MgSO_4$, 6.67 g $CaCl_2$-$2H_2O$, 0.019 g $(NH_4)_6Mo_7O_{24}$-$4H_2O$, 0.198 g $FeSO_4$-$7H_2O$, and 100 mL of "Metals 44" (2.5 g EDTA free acid, 10.95 g $ZnSO_4$-$7H_2O$, 1.54 g $MnSO_4$-H_2O, 0.392 g $CuSO_4$-$5H_2O$, 0.250 g $C(NO_3)_2$-$6H_2O$, 0.177 g $Na_2B_4O_7$-$10H_2O$, 100 µL H_2SO_4 dissolved in 1 L dH_2O). pH was adjusted to 6.8 with up to 100 mL of 1 M KOH in dH_2O and added dH_2O to a total volume of 1000 mL.

We prepared Luria Bertani media (LB) using a recipe of 10 g tryptone (US Biological Life Sciences), 5 g yeast extract (BD and later US Biological Life Sciences yeast extract), and 10 g NaCl (Fisher Scientific) per liter of H_2O. We prepared LB agar plates by adding 7.5 g BD Bacto™ Agar per 500 mL LB broth. Volumes of antibiotics including kanamycin and streptomycin were added to LB and LB agar to bring

concentrations to 10 µg/mL and 20 µg/mL, respectively. We streaked mutants on LB-kan^{10} plates and plated dilution series on LB-str^{20} plates.

In the second portion of our experiment, we tested transformation of ADP1/BD413 in varying liquid environmental conditions and temperature variations, including LB with added succinate, LB deficient in iron using an iron chelator, LB in which the Na^+ was replaced with equimolar K^+ (LBK), LBK with added succinate, LBK deficient in iron using an iron chelator, LB with both succinate and the iron chelator, LB with succinate and supplemented with divalent cations, "Combination" media consisting of LB with succinate, the iron chelator, and the divalent cations, and LB with succinate at 20C, 27C, and 30C. Recipes for these test broths are as follows.

In all cases final liquid volumes are assumed to be 1 L. *LBK*: 5 g yeast, 10 g tryptone, 17.7 g Sigma-Aldrich KCl, and 1 L H_2O. *LBK+succinate*: LBK with 0.02% succinate. *LBK+Fe-deficient*: LBK with 20 µM 2,2-dipyridyl. *LB+succinate*: LB with 0.02% succinate. *LB+succinate-Fe-deficient*: LB with 0.02% succinate and 20 µM 2,2-dipyridyl. *LB+succinate+divalent cations*: LB with 0.02% succinate, 2 mM $MgSO_4$, and 100 µM $CaCl_2$.

DNA isolation:

Crude lysate was isolated from mutants obtained from the French collection using a heat-lyse protocol. We grew overnight cultures of mutant cells in LB-kan^{10}, pelleted, resuspended in sterile water, and heated samples to lyse; see **Moving *ΔCIADxxxx::kan mutations* into the wild type background** for more detailed protocol. We moved mutations from the French collection into a fresh, highly competent wild ADP1

background to ensure competency, as *A. baylyi* lose competency when left on plates for extended periods of time (Bacher *et al*, 2006).

A. baylyi does not require sequence specificity in template DNA for transformation, which means it may uptake and integrate any form of double-stranded exogenous DNA. However, rates of recombination decrease with increased DNA sequence divergence (Thomas and Nielsen, 2005). We isolated chromosomal DNA from a streptomycin-resistant strain to use as a transformation marker. The streptomycin-resistant (str^R) strain of ADP1 was obtained from Bruce Voyles of Grinnell College. After culturing these str^R cells in 12 mL of LB-str^{20} broth overnight, we pelleted the cells and resuspended them in 1 mL of saline (10 g/L NaCl). We split the total volume into two microfuge tubes and added 500 µL of 25 phenol-24 chloroform-1 isoamyl alcohol to each tube. To each microfuge tube, we added 50 µL 3 M sodium acetate (CH_3COONa) to a final concentration of 0.3 M and 1000 µL of 200 proof ethanol (CH_3CH_2OH). We incubated for 1 hour at -70C to precipitate DNA and nucleic acids and then evaporated off supernatant at 37C. Lastly, we resuspended the contents of each microfuge tube in 100 µL in Qiagen EB buffer, composed of 10 mM Tris-Cl of pH 8.5. The Nanodrop reading of final concentration of crude DNA was 1066.7 ng/µL with a 260/280 of 1.64.

Moving *ACIADxxxx::kan mutations* into the wild type background (*Competence Relative to the Functional Significance of Type IV Pilus Proteins)*:

We made streak plates of wild type ADP1 cells from freezer stock on *Acinetobacter* minimal succinate agar and incubated overnight at 37C. In the same way, we made streak plates of the *ACIADxxxx::kan* mutants from the French collection freezer

stock on LB-kan^{10} (10 µg/mL kanamycin) and incubated overnight at 37C. We parafilmed and stored the streak plates in the refrigerator, keeping them for no longer than a week. We grew overnight cultures of both wild type and mutant ADP1 cells by inoculating 2 mL test tubes of LB and LB-kan^{10}, respectively. We incubated cultures overnight with high aeration at 37C. The following day, we pelleted 1.5 mL of the *ACIADxxxx::kan* mutant cells in a microfuge tube. Cells were resuspended in 100 µL of sterile water and heated at 95C in a heat block for 2 hours to lyse cells and release genetic content.

After lysing, we placed the cell lysates on ice and obtained an LB plate for each *ACIADxxxx::kan* mutant to move mutations into the wild type. We also created a sterility control LB plate, on which we plated 5 µl samples of each mutant's cell lysate to incubate overnight at 37C to assess sterility. On an LB plate for each mutant, we placed 50 µL of the wild type overnight culture in the middle of the plate and added 10 µL of lysate from the respective *ACIADxxxx::kan* mutant to the "puddle." "Puddles" were allowed to soak into the LB agar and dry, aided by a Bunsen burner, and plates were incubated overnight at 37C. The following day, we evaluated the sterility control LB plate for growth or lack thereof, which ensured cell lysates were sterile. We scraped off the cell "puddle" and transferred the sample into 750 µL of saline (10 g/L NaCl), pipetting and vortexing to mix thoroughly. We performed a ten-fold dilution series from 10^0-10^7 in a microtiter plate, diluting with saline (10 g/L NaCl). See **Appendix 2: Performing a ten-fold dilution series**. We plated the ten-fold dilution series on LB-kan^{10} agar, incubating overnight at 37C. See **Appendix 2: Plating ten-fold dilution series**. The following day, dilutions exhibiting well-isolated single colonies were selected

to make streak plates on LB-kan^{10} agar. We made two streak plates for each mutant and incubated overnight at 37C. Once again, we selected single colonies from each mutant streak plate and re-streaked on a new, final LB-kan^{10} plate and incubated overnight at 37C.

We prepared two tubes of -80C frozen glycerol stocks of each new mutant strain from these streak plates as follows: We placed 1.5 mL of filter-sterilized 25% glycerol LB in a cryogenic vial and inoculated each with a long-stem Q-tip containing all cells from the final streak plates. One cryogenic vial was prepared for each mutant plus one additional vial as a duplicate. We stored the cryogenic vials at -80C and recorded new strain numbers per the Lostroh lab in the laboratory strain book.

Puddle transformation on solid media (*The Functional Significance of Type IV Pilus Proteins for Competence)*:

We grew wild type and mutant *A. baylyi* to competence as follows: We streaked out wild type ADP1 cells on minimal succinate plates and grew colonies overnight at 37C. We streaked out mutant ADP1 cells on kan^{10} plates and grew colonies overnight at 37C. We parafilmed and stored the streak plates in the refrigerator, keeping them for no longer than a week. The following day, we grew an overnight culture of a colony of *A. baylyi* in 2 mL LB and in parallel, grew an overnight culture of *A. baylyi* mutant in 2 mL LB-kan^{10}, both with high aeration at 37C. We tested competence of mutant bacteria compared to wild type BD413/ADP1. To 50 µL of each cell culture, both mutant and wild type, we added 5 µL of isolated str^{R} DNA and pipetted several times to mix thoroughly. We transferred a total of 50 µL of cell culture with str^{R} DNA to an LB plate, allowed “puddle”

to soak in and dry, with the aid of a Bunsen burner, and incubated overnight at 37C. The following day, we scraped off the cell "puddle" and transferred the sample into 750 µL of saline (10 g/L NaCl), pipetting and vortexing to mix thoroughly. We performed a ten-fold dilution series from 10^0-10^7 in a microtiter plate, diluting with saline (10 g/L NaCl); see **Appendix 2: Performing a ten-fold dilution series**. We plated the ten-fold dilution series in parallel on both LB plates and LB-str^{20} plates, allowed plates to soak in and dry, and incubated the plates overnight at 37C; see **Appendix 2: Plating ten-fold dilution series**. The following day, we calculated transformation efficiency as a ratio of transformed cells/mL to total cells/mL; Calculate total cells/mL for each "puddle": [(average colonies on LB)/(10 µL droplet)] x (dilution factor) x (1000 µL/1 mL). We compared transformation efficiency of the mutants to that of the wild type.

Evaluating efficacy of different liquid media on natural transformation (*Effects of Environmental Conditions on Competence in* Acinetobacter baylyi):

We streaked wild type ADP1 cells on minimal succinate plates and grew them overnight at 37C. We inoculated 4 ml of LB broth with a single colony and grew overnight with high aeration at 37C. This is henceforth referred to as *overnight culture* and serves as the inoculum. We added 1 ml of overnight culture to 24 mL of *LB control broth* or *test broth* in a side arm flask.

When we performed each experiment, a LB control was grown alongside the test broth with high aeration at 37C and later, varying temperatures of LB+succinate at 27C, 30C, and 37C. Both contained the same overnight culture inoculum. After inoculation of both LB control and test broth, we obtained OD600 of both LB control broth and test

broth every half hour beginning at 2 hours. Using these spectrophotometric results, we produced a growth curve, comparing the rate of growth of the LB control to that of the test broth.

This growth curve allowed us to determine proper time to test for transformation efficiency, defined as *sampling time*. When OD600 of the test broth reached that of the LB control at 3 hours, we removed cells to sample and test transformation efficiency. Prior, unpublished research from the Lang and Lostroh laboratory indicates that transformation efficiency of ADP1 achieves its maximum in LB at 37C at 3 hours (Figure 6).

We removed 0.5 mL of cells at 3 hours for LB control broth and sampling time for test broth, transferred to a sterile microfuge tube, and added 5 µL of isolated str^R ADP1 DNA. Cells were incubated with DNA at 37C for 1 hour. To degrade extracellular DNA after this incubation, we added 55 µL of New England BioLabs Inc. 10X DNase I digestion buffer (10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ at pH ~7.6) and 5 µL of New England BioLabs Inc. DNase I (5 mg/mL in 0.15 M NaCl). We incubated cells treated with DNase and digestion buffer at 37C for 15 minutes. We performed a ten-fold dilution series from 10^0-10^7 in a microtiter plate, diluting with saline (10 g/L NaCl), and plated on LB agar and LB-str^{20} selective agar. We allowed plates to soak in and dry, with the aid of a Bunsen burner, and incubated the plates overnight at 37C. The following day, we determined transformation efficiency, defined as ratio of transformed cells/mL to total cells/mL. Transformation efficiency of the test broth was compared to that of the LB control broth.

Results.

The Functional Significance of Type IV Pilus Proteins for Competence:

To test competence of *tdk-kan* knockouts, we cultured both wild type control and mutant colonies in LB broth and LB-kan^{10} broth, respectively. We added DNA conferring str^{R} to samples of each of these overnight cultures and plated samples with DNA in a "puddle" on an LB plate for transformation. The following day, we transferred "puddle" to sterile saline and performed a ten-fold dilution. We plated this ten-fold dilution in parallel on LB to determine total cells/mL and on LB-str^{20} to determine transformed cells/mL.

Transformation efficiency is the measure of a bacteria's ability to collect exogenous DNA, import it DNA across the periplasm, and incorporate it into its own genome, therefore it is a measure of competence. Transformation efficiency is a ratio of transformed cells/mL to total cells/mL. Mutants lacking proteins ComM, PilF, PilC, PilU, PilT, ComA, ComP, PilV, FimT, and ComC had no detectable transformation. The detection limit is calculated as a maximum of one colony per total number of cells/mL plated, so the detection limit in these experiments ranged from 4.25×10^{-10} to 6.7×10^{-10} (1/total number of cells on plain LB). Note that mutants are named for whatever gene is lacking, as each mutant has the coding sequence for the targeted gene completely replaced with a kanamycin-resistance gene. The results suggest that these proteins are integral to either the formation of the competence appendage or essential in the import and recombination of DNA into *A. baylyi*'s genome. Mutants lacking proteins ComEA, CHP (ACIAD3352), PilG, PilR, PilS, ComF, and ComE exhibited detectable transformation (Figure 5). These data suggest that these proteins are less essential to

either the competence appendage's formation or the import and recombination of DNA into *A. baylyi*'s genome. Mutants' transformation efficiency was compared to that of a fully functional wild type *A. baylyi* of mean transformation efficiency 2.68 X 10^{-3}, or a log transformation efficiency of -2.57. Transformation efficiency is the proportion of transformed cells so that on the log scale, 0 corresponds to a ratio of 1, or all of the cells having been transformed.

Notably, the *comP* mutant demonstrated no detectable transformation, which was expected and served as a control. ComP is the major pilin that comprises the T4P and was already known to be involved in DNA binding and uptake (Porstendörfer *et al,* 1997 and Porstendörfer *et al*, 2000).

Secondly, similar to ComP, ComC had no detectable transformation – it was not competent whatsoever. Link *et al* demonstrated that ComC is essential for natural transformation in ADP1 (1998). It has a leader sequence for secretion and is homologous to certain T4P biogenesis proteins, adhesins. ComC is likely found to be associated with the pilus tip, acting as a competence factor involved in DNA binding and uptake. ComC is similar to proteins that aid in the assembly of T4P in pathogens and has an N-terminus that is reminiscent of a signal peptide (Link *et al*, 1998). The Averhoff laboratory found that a *comC* mutant was unable to bind or take up DNA in liquid media, but that ComC was unnecessary for the biogenesis of pilus fibers in *A. baylyi* grown in liquid.

Compellingly, *comC* is understood to exist in an operon with *comF*, *comE*, *comC*, *pilX*, *comB*, *pilV*, and *fimU* from ACIAD3314-3321 (see Figure 6). Yet *comE*'s mutant exhibited some competence, log transformation efficiency of -3.26 compared to that of wild type, -2.57.

***Effects of Environmental Conditions on Competence in* Acinetobacter baylyi:**

To test competence of ADP1 in LB control and test broth, we began by culturing ADP1 in LB broth to create an overnight broth to serve as the inoculum. We inoculated the LB control broth and four replicates of the test broth in side arm flasks in a 1:25 ratio. We obtained OD600 of both LB control broth and test broth to evaluate cell growth every half hour beginning at 2 hours. Once OD600 of test broth matched that of the LB control broth at 3 hours, we sampled the broths and tested each cell population for competence as follows. We treated cells with antibiotic-resistance marker DNA and incubated for one hour. To stop DNA uptake, we added DNase digestion buffer and DNase and incubated for 15 minutes. Then, we performed a ten-fold dilution series on general-purpose LB agar and selective LB-str[20] agar to determine transformation efficiency, defined as the ratio of transformed cells/mL to total cells/mL.

Figure 8 presents the transformation efficiency and growth rate of *A. baylyi* grown in LB with a variety of test broths.

Bacterial reproduction is an appropriate measure of overall cellular health and has been linked to induction of natural competence. Published studies have demonstrated that *A. baylyi* is most competent at the transition to log-phase growth (Bacher *et al*, 2006). Prior, unpublished research from the Lang and Lostroh lab indicates that transformation efficiency of ADP1 achieves its maximum at 3 hours. This indicates that cells have grown to an ideal density in which environmental and biochemical cues are right for competence to take place (Seitz and Blokesch, 2013); the optical density of ADP1 at 600 nm under these conditions ranged from 0.11 to 0.21 absorbance units. Therefore, for all of the experiments testing different environmental conditions, we measured competence

when the cells under test conditions achieved the same OD600 as that achieved at 3 hours by cells grown under the control conditions (in LB and 37C with aeration). Most notably in our experiment, the population in test broth LB+succinate increased at a greater rate than cells in the LB control and so cells grown under these conditions were tested for competence at 2.75 hours sampling time (when they had already achieved an OD600 of at least 0.42 absorbance units). Addition of succinate was the only experimental condition in which we tested competence prior to 3 hours, indicating that cells grown in a succinate-enriched environment are more metabolically active in response to their supplemented environment. In contrast to LB+succinate, we sampled cells grown in LB+Fe-deficient and LBK+Fe-deficient at 3.5 hours post inoculation, because it took them longer to achieve an OD600 of 0.39 and 0.33 absorbance units, respectively. These results indicate that an iron-poor environment results in slower bacterial growth. Finally, when we evaluated cells grown in LB+succinate at 20C, sampling time was 6 hours because the lowered temperature resulted in much slower population growth to achieve an OD600 of 0.36 absorbance units.

LB+succinate:

We suspected added succinate might enhance competence for two reasons. First, succinate is a substrate necessary for carbon catabolite repression of multiple operons (Fischer *et al*, 2008). Carbon catabolite repression is a mechanism in *A. baylyi* that allows for efficient use of carbon sources (Fischer *et al*, 2008). Second, suberin is a complex plant polymer comprised of hydrocarbons and aromatic carbons that is secreted in response to plant stress, and degraded suberin yields succinate (Young *et al*, 2005). *A. baylyi* are naturally occurring in soil, in which plant matter and hence, suberin is

abundant. Growth with succinate provides bacteria with more familiar, plant-derived carbons of its natural habitat. For both reasons we suspected that supplementing an already rich environment with succinate might increase all cellular metabolic activity and thus, increase competence.

In agreement with this hypothesis, of all conditions tested, LB+succinate produced the greatest transformation efficiency. We found that cells grown in LB+succinate were 2.5 times more competent than LB control cells ($p = 0.324$ using a statistical t-test). Further, cells grew at a faster rate than the LB control broth, indicating that succinate is a nutritious additive to optimize environmental and biochemical cues to induce competence.

LB+Fe-deficient:

We suspected that removing iron from ADP1's environment might trigger a response to competence regulation. Whether that response to competence regulation would be positive or negative was unknown, but iron deficiency was tested for several reasons. Most bacteria import reduced Fe^{2+} to utilize it in the quaternary structure of enzymes; the reduced iron is often used as a cofactor for enzymatic activity. Further, reduced Fe^{2+} is the form of iron that circulates the human bloodstream – a source of iron for pathogens such as *A. baumannii*, *A. baylyi*'s cousin. Anemia is an innate immune response to an infection, as Fe^{2+} is pulled out of circulation in an attempt to sequester it from pathogens. In response to this, pathogens have evolved virulence gene express upon entering an iron-depleted environment (Doherty, 2007). Because of *A. baylyi*'s relatedness to pathogens, we sought to determine whether it might regulate competence in response to iron. We added an iron chelator to our LB broth to create an artificially

iron-lacking environment. This theory did not produce the hypothesized increase in competence, rather we found that cells grown in LB+Fe-deficient test broth were 1.5 times less competent than LB control cells ($p = 0.0373$ using a statistical t-test).

Considering growth rates in this iron deficient environment, all bacteria, including *A. baumannii* and *A. baylyi* require essential nutrient metals such as iron, zinc, manganese, etc. that serve as co-factors for many vital processes (Mortensen and Skaar, 2013), so we expect that lack of environmental nutrient iron stunts cellular growth. Iron restriction with 2,2-dipyridyl is known to inhibit growth of *A. baumannii in vitro* (de Léséleuc *et al*, 2012). So not surprisingly, cells grew slower in the iron deficient broths, sampling time was 3.5 hours post-inoculation for LB-Fe deficient and LBK-Fe deficient.

LBK:

The only difference between general-purpose LB and LBK is an alteration in monovalent cation identity. LB contains sodium chloride, NaCl, while LBK contains potassium chloride, KCl. Comparing LB to LBK allows us to evaluate whether a difference in monovalent cation presence, K^+ instead of Na^+, might alter transformation efficiency. We found that cells grown in LBK test broth were a full order of magnitude less competent than the cells grown in LB control broth ($p = 0.481$ using a statistical t-test) even though they achieved an OD600 of 0.24 absorbance units in the same amount of time as cells grown in LB.

After testing single changes in environmental additives, we tested combinations of LB+succinate, LB+Fe-deficient, and LBK.

LB+succinate+Fe-deficient and LBK+succinate

When added individually to LB, succinate increases the rate of natural transformation. When added individually, iron deficiency or LBK each decrease the rate of natural transformation. Given this we might expect that combining either iron deficiency or LBK with succinate in LB broth should produce an intermediate rate of transformation similar to the control. This is in fact what happens. The positive effect of the succinate and the negative effect of the iron deficiency or LBK somewhat neutralize each other.

LBK+Fe-deficient:

Individually, both LBK and iron deficiency decreases the rate of natural transformation. Given this we might expect that combining to two would result in a net decrease in competence. This does in fact happen, but strikingly, cells grown in LBK-Fe deficient test broth resulted in a rate of transformation 1000 times less than that of LB control cells ($p = 0.000195$ using a statistical t-test). The negative effects of both LBK and iron deficiency combined result in a constructive net negative effect on competence greater than that of LBK and iron deficiency alone. Compellingly, cells grown in LBK+Fe-deficient did not grow 1000 times slower than control cells. The cells in this test broth achieved an OD600 of 0.35 in 3.5 hours.

LB+succinate+divalent cations:

Prior research suggests that natural transformation is dependent on divalent cation presence to aid in DNA binding. In *A. baylyi* cultures treated with EDTA to remove divalent cations, natural transformation was totally repressed (Palmen *et al*, 1993). Further, competence could be restored, albeit to 7% of their previous levels with the

addition of 5 mM Mg^{2+} and Ca^{2+} (Palmen *et al*, 1993). Given these results, we expected adding divalent cations to increase transformation rates.

In contrast to our expectation, we found that the cells grown in LB-succinate with divalent cations test broth were 2 times less competent than LB control cells ($p = 0.106$ using a statistical t-test). Since the addition of the succinate was shown to have a positive effect on transformation, we conclude that adding divalent cations had a negative effect on the rate of natural transformation.

Temperature:

Figure 9 presents the transformation efficiency of *A. baylyi* grown in LB+succinate at a variety of temperatures. We chose this temperature range because prior research suggests that *A. baylyi* grows optimally *in vivo* between 30-37C, but can also grow slowly at room temperature (Metzgar *et al*, 2004). In addition, because it is a soil bacterium, *A. baylyi*'s natural, open-air environment is much colder than the typical *in vitro* culture temperature. We wanted to also look at temperatures more akin to its natural habitat. At all temperatures, we grew *A. baylyi* in LB+succinate, since that was the tested condition yielding the maximum transformation efficiency. Therefore, we postulated that ADP1 might have a heightened transformation efficiency if incubated at a temperature more akin to its natural habitat. Furthermore, the *Acinetobacter* species is known to survive at a wide range of temperatures (Chen *et al*, 2008).

Cells grown at 20C were 1.6 times less competent than control cells ($p = 0.752$ using a statistical t-test). Cells grown at 27C were 1.5 times less competent than control cells ($p = 0.432$ using a statistical t-test). Cells grown at 30C were 21.5 times less competent than control cells ($p = 0.185$ using a statistical t-test). Competence seemingly

dips around 30C, yet transformation efficiencies at lower temperatures are in the same order of magnitude of that of cells grown at 37C. However, sampling time was 6 hours and 3.75 post-inoculation for 20C and 27C, respectively, indicating slower growth. In contrast, sampling time for cells grown at 30C was 3 hours, which is the same as that for control cells at 37C.

Discussion.

The Functional Significance of Type IV Pilus Proteins for Competence:

The results of our experiments elucidated which genes and their respective proteins are required for competence to take place. Our hypothesis stated that if a protein helped comprise or was directly related to the hypothetical competence machine was knocked out, the subsequent mutant would not be competent. The majority of genes we tested with a novel protocol to quantify natural transformation on a solid agar surface were deemed indispensable to competence. Available literature combined with Leah Lieber's 2014 thesis data and work performed by Rebecca Bloomfield as compiled to construct both a theoretical competence pilus and twitching pilus for *A. baylyi* (see Figure 4). All tested genes with the exception of *comEA*, *CHP* (ACIAD3352), *pilG*, *pilR*, *pilS*, *comF*, and *comE* in *A. baylyi* were necessary for competence.

Herzberg *et al* identified ComB, ComC, ComE, ComF, and ComP as necessary for competence in broth (2000). Our results for *comC* and *comP* concur with those of the Herzberg lab – these two *tdk-kan* knockouts yielded no rate of transformation. However, contrary to Lieber and Herzberg *et al*'s (2014 and 2000) findings, we found that ComE and ComF are not indispensible for competence, as we detected evidence of transformation. Herzberg *et al* proposes that ComE, ComF, ComP, and ComB together

build a heteropolymeric structure responsible for mediating DNA uptake (2000). Complicating the matters further, ComC, ComF, and ComE all coexist within an operon comprising ComF, ComE, ComC, PilX, ComB, PilV, and FimU in the respective order (see Figure 6). We question why ComE results in some competence, though nearly a full order of magnitude less competent than wild type, as the literature and available information on ComE supports the contrary. However, Lieber finds that ComE is required for twitching; there is no published data on ComE with regard to twitching. We suspect that ComE is some sort of minor pilin subunit in the external appendage.

Compellingly, Porstendörfer *et al* found that a *comE* deletion resulted in anatomically correct and functional pili in broth (1997). This held true for *comB*, *comE*, *comF*, and *comP* and researchers suggested that the pilus structure itself is not involved in transformation in broth.

Yet, in concurrence with Lieber, the ComP knockout mutant exhibited no detectable transformation (2014). Interestingly, Porstendörfer *et al* found that a *comP* deletion results in a anatomically correct and functional pilus (1997). Our laboratory, however, defines ComP as a major pilin component comprising a large potion of the external pilus structure (see Figures 3 and 4).

Porstendörfer *et al* found that there is significant amino acid similarity between ComP and pilins, the structural subunits of T4P (1997). Both ComP and other pilins have a conserved short leader peptide, a cleavage motif for an endopeptidase, a hydrophobic N-terminal domain, and a pair of cysteine residues near the carboxy terminus and a characteristic size of 145-160 amino acids. Published research suggests ComP is the

major pilin structural subunit for the thick T4P, and it undergoes glycosylation and is located in both the cytoplasmic and outer membranes (Porstendörfer *et al*, 1997).

In spite of ComP's necessity in competence, the Averhoff laboratory found no effect on piliation in liquid media, as mutants with mutations in one or more of the competence genes *comB*, *comE*, *comF*, and *comP* all had anatomically correct and functional pili in their experiments (Porstendörfer *et al*, 1997). They therefore suggested that pilin-like ComP is actually unrelated to the pilus-like structure, and instead that ComP functions as a major subunit of an organelle that acts as a channel or pore mediating DNA binding and/or uptake. Their experiments were done using very different conditions compared to ours.

The Averhoff lab further reported that *comP* expression is not concurrent with competence induction, as transcription of *comP* is highest in the late stationary growth phase and competence immediately begins upon inoculation of stationary-phase culture (what we call "overnight culture") into fresh medium. The fact that *comP* expression increases during sustained exponential growth and stationary phase independent of transformation frequencies indicates that the DNA uptake nanomachine exists before the maximal competence point, allowing cells to instantly take in DNA when environmental conditions are auspicious. Perhaps ADP1 cells have pre-existing pili external to the cell body composed of only ComP units and when conditions for competence are ripe, cells may build the remaining pilus with minor pilins (including FimT, ComE, ComF, and PilV and proteins not tested PilX, FimU, and ComB) and finally the ComC pilus tip.

Chen and Dubnau identified ComC as the adhesion protein at the tip of the pilus (2004). Lieber found that ComC is required for both competence and twitching, but does

not result in a total lack of twitching (2014). Lieber's competence results mirror that of published data, but literature suggests that ComC is not needed for twitching. Link *et al* found that ComC was not needed for twitching, as respective knockout mutants had a piliation phenotype identical to the wild type (1998). However, they did not test twitching using a surface-based protocol. They also found ComC to be unnecessary for pilus fiber biogenesis in ADP1.

ComC is a competence factor involved in DNA binding and uptake. It is an ortholog to K02674 T4P assembly protein PilY1 and orthologs often, but not always, retain the same function. Because T4P facilitate twitching, a protein involved in the assembly of T4P would be essential for normal twitching to occur. Consequently, a mutant lacking a gene involved in T4P assembly would be deficient in twitching because its T4P would not be properly and/or effectively assembled.

In compilation with twitching data acquired by Leah Lieber and Rebecca Bloomfield, we determined which genes are required for competence, twitching or neither. ComP is required for competence but not for twitching. PilC is required for both competence and twitching. ComA is required for competence but not for twitching. ComEA is not required for competence or twitching. ComF is not required for competence but it is unknown if required for twitching. PilV is required for competence and somewhat required for twitching. PilF is required for both competence and twitching. FimT is required for competence but not for twitching. ComM is required for both competence and twitching. PilU is required for competence but not for twitching. PilT is required for competence but not for twitching. ComC is required for competence but not for twitching. ComE is not required for competence but required for twitching. CHP is

neither required for competence nor twitching. PilG is neither required for competence nor twitching. PilR is not required for competence but it is unknown if required for twitching. PilS is neither required for competence nor twitching.

Lieber's thesis most remarkably found that there exists a very strong positive relationship between twitching and transformation ability, R = 0.85737 (2014). Mutants with greater twitching ability were also more competent, alluding to a strong probability that there is a physiological communication between the two. Based on available data on ADP1 knockout mutants, we have constructed hypothetical figures of both the competence and twitching pili. Proteins were structured based on relative importance in relation to competence or twitching data.

***Effects of Environmental Conditions on Competence in* Acinetobacter baylyi:**

Moving into the latter experiment on maximal competence conditions in LB, it is important to note that the entire *Acinetobacter* genus, including ADP1, is capable of exploiting a number of carbon and energy sources to thrive. Members of the *Acinetobacter* genus are naturally found in varying environments, including soil, water, human skin, and mucous membranes (Manchanda *et al*, 2010). Though *A. baumannii* is the most clinically relevant species of *Acinetobacter*, ADP1's benign properties and high frequency of transformation afford researchers the ability to study natural transformation. Yet, selective pressures, most saliently antibiotic resistance, have allowed for the fittest of both species to survive and thrive. Newer research indicates that *A. baylyi*'s novel pathogenicity is also resistant to available antibiotics (Chen *et al*, 2008). Our research has

demonstrated that *A. baylyi* is substantially adaptable – ADP1 are competent in a variety of *in vitro* LB-based conditions.

Perhaps ADP1's environmental adaptability affords ability for stability in growth patterns and transformation efficiency. *A. baylyi* are soil-dwelling bacteria; they are found in soil, water, and sewage. All of these natural environments are incredibly variable, containing different chemical makeups at any given time. Therefore, *A. baylyi*'s nutrient availability is dependent on its surroundings, suggesting an evolutionary necessity to quickly adapt to novel environmental compositions. As mentioned prior, *A. baylyi*'s naturally transformative properties are advantageous: to acquire fitness-enhancing traits (i.e. genes conferring antibiotic resistance), to serve as a template in DNA repair, and finally, as a source of carbon, nitrogen, and phosphorous.

Bacteria are capable of employing many different compounds as carbon and energy sources to grow and reproduce. They may utilize a diverse array of metabolic pathways to process hydrocarbons, aliphatic alcohols, glycols and polyols, etc. to respond to changes in environmental pressures.

Members of the *Acinetobacter* genus are renowned for their metabolic versatility and ability to adapt to changing environmental pressure. Our findings corroborate this statement, as *in vitro*, we most notably found that infusing general-purpose LB with succinate or iron chelator regulates competence positively or negatively, respectively. Yet overall, *A. baylyi* strain ADP1 are incredibly competent in various tested broths.

A. baylyi's growth hardiness and ease of cultivation as demonstrated by this study underscore its capability as a model organism. Its similarity to *A. baumannii* and coupled with its own emergent traits, *A. baylyi* is a compelling organism to study. By determining

ideal *in vitro* conditions in which to culture ADP1 cells for maximized competence, we can provide other *A. baylyi* and even *A. baumannii* researchers with the tools to develop further experiments involving physiological characterization and metabolic activity.

The addition of succinate to LB broth only slightly upregulates competence, suggesting that the ADP1 cells our laboratory and other research labs obtain from the ATCC have been selected to grow in typical *in vitro* conditions and adapted to metabolize carbon sources beyond their natural ones. Secondly, the infusion of an iron chelator to an LB broth with sodium replaced by potassium yielded the most drastic change in competence. LBK+Fe-deficient actually decreased competence by 1000 times, suggesting a potential LB combination that effectively turns natural transformation off. Our results reiterate that ADP1 are hardy organisms, thriving under a variety of environmental conditions. Our results found that ADP1 cells are competent across all tested environmental conditions, but slightly more competent in LB+succinate and severely less competent in LBK+Fe-deficient.

Though our studies aim to verify and define necessary protein units for competence to occur and subsequently, determine the ideal *in vitro* conditions and temperatures in which ADP1 cells might be most competent, competence involves four major events: first, competence induction, second, DNA binding, third, DNA import, and fourth, integration and expression of genetic material (Palmen and Hellingwerf, 1996; Averhoff, 2004). It is difficult to isolate discrete steps, as drastic environmental changes might affect each of these events discretely, offering room for conflation of the four. Our use of a streptomycin marker in our DNA equates a competence to full expression of the streptomycin gene. Ideally, we might design an experiment in which transformation

efficiency and level of expression of competence are correlated. Others have found success in designing *Bacillus subtilis* strains with both wild type *comK* and an additional *comK*::*lacZ* fusion integrated in the chromosome at the *amyE* locus to relate both transformation efficiency and expression of competence by evaluating b-galactosidase activity (Palmen *et al*, 1993 and Palmen 1994; data not shown). We could create similar *A. baylyi* reporter gene fusions to visualize the translocation of DNA during competence.

Next, we might confirm our hypothesized competence and twitching nanomachine structures by employing fluorescent protein fusion to confirm relative protein localization. Such a future experiment would also allow us to determine when in the ADP1 growth cycle that particular genes are expresses and proteins translated. Because it is understood that the time required for a cell to make a single competence nanomachine exceeds that of a single generation, it will be compelling to see when during the ADP1 growth cycle the competence pilus is constructed.

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Works Cited.

Alyamani E, Khiyami MA, Booq RY. 2014. *Acinetobacter baylyi* biofilm formation dependent genes. J. Pure Appl. Microbio. **8**(1):379-382.

Averhoff B. 2004. DNA transport and natural transformation in mesophilic and thermophilic bacteria. J. Bioenerg. Biomembr. 36(1):25-33.

Averhoff B and Graf I. 2008. The natural transformation system of *Acinetobacter baylyi* ADP1: A unique DNA transport machinery. *Acinetobacter Molecular Biology*, 120-139.

Bacher JM, Metzgar D, de Crécy-Lagard V. 2006. Rapid evolution of diminished transformability in *Acinetobacter baylyi*. J. Bacteriol. **188**(24):8534-8542.

Barbe V, Vallenet D, Fonknechten N, Kreimeyer A, Oztas S, Labarre L, Cruveiller S, Robert C, Duprat S, Wincker P, Ornston LN, Weissenbach J, Marliére P, Cohen GN, Médigue C. 2004. Unique features revealed by the genome sequence of Acinetobacter sp. ADP1, a versatile and naturally transformation competent bacterium. Nucleic Acids Res. **32**(19):5766-5779.

de Berardinis V, Vallenet D, Castelli V, Besnard M, Pinet A, Cruaud C, Samair S, Lechaplais C, Gyapay G, Richez C, Durot M, Kreimeyer A, Le Févre F, Schächter V, Pezo V, Döring V, Scarpelli C, Médigue C, Cohen GN, Marliére P, Salanoubat M, Weissenbach J. 2008. A complete collection of single-gene deletion mutants of Acinetobacter baylyi ADP1. Mol. Syst. Biol. **4**:174. doi:10.1038/msb.2008.10.

Burrows LL. 2005. Weapons of mass retraction. Mol. Microbiol. **57**(4):878-888.

Burrows LL. 2012. Prime time for minor subunits of the type II secretion and type IV pilus systems. Mol. Microbiol. **86**(4):765-769.

Chen I, Christie PJ, Dubnau D. 2005. The ins and outs of DNA transfer in bacteria. Science. **310**(5753):1456-1460.

Chen TL, Siu LK, Lee YT, Chen CP, Huang LY, Wu RCC, Cho WL, Fung CP. 2008. *Acinetobacter baylyi* as a pathogen for opportunistic infection. J. Clin. Microbiol. **46**(9):2938-2944.

Chen I, Dubnau D. 2004. DNA uptake during bacterial transformation. Nature Rev. Microbiol. **2**:241-249.

Craig L, Pique ME, Tainer JA. 2004. Type IV pilus structure and bacterial pathogenicity. Nature Rev. Microbiol. **2**:363-378.

Davies J, Davies D. 2010. Origins and evolution of antibiotic resistance. Microbiol. Mol. Biol. Rev. **74**(3):417-433.

Doherty C. 2007. Host-Pathogen Interactions: The Role of Iron. J. Nutri. **137**(5):1341-1344.

Evans LD, Poulter S, Terentjev EM, Hughes C, Fraser GM. 2013. A chain mechanism for flagellum growth. Nature. **504**(7479):287-290.

Fischer R, Bleichrodt FS, Gerischer UC. 2008. Aromatic degradative pathways in *Acinetobacter baylyi* underlie carbon catabolite repression. Microbiology. **154**(10):3095-3103.

Fournier PE, Richet H. 2006. The epidemiology and control of *Acinetobacter baumannii* in health care facilities. Clin Infect. Dis. **42**(5):692-699.

Gohl O, Friedrich A, Hoppert M, Averhoff B. 2006. The thin pili of *Acinetobacter* sp. strain BD413 mediate adhesion to biotic and abiotic surfaces. Appl. Environ. Microbiol. **72**(2):1394-1401.

Herzberg C, Friedrich A, Averhoff B. 2000. *comB*, a novel competence gene required for natural transformation of *Acinetobacter* sp. BD413: identification, characterization, and analysis of growth-phase-dependent regulation. Arch Microbiol. 173(3):220-228.

Harrell KF, McBride MJ. 2008. The surprisingly diverse ways that prokaryotes move. Nature Rev. Microbiol. **6**:466-476.

Juni E. 1974. Simple genetic transformation assay for rapid diagnosis of *Moraxella osloensis*. Appl. Microbiol. **27**(1):16-24.

Korotkov KV, Sandkvist M, Hol WGJ. 2012. The type II secretion system: biogenesis, molecular architecture and mechanism. Nature Rev. Microbiol. **10**:336-351.

Lang K, Lostroh P. 2013. RUI: Biology-Physics Collaboration to Investigate the Genetic and Structural Basis of Competence in Bacteria. Unpublished observations.

de Léséleuc L, Harris G, KuoLee R, Chen W. 2012. Antimicrob. Agents Chemother. **56**(10):5397-5400.

Link C, Eickernjäger S, Porstendörfer D, Averhoff B. 1998. Identification and characterization of a novel competence gene, *comC*, required for DNA binding and uptake in *Acinetobacter* sp. strain BD413. J. Bacteriol. 180(6):1592-1595.

Lieber L. 2014. Functional significance of type IV pilus proteins in *Acinetobacter baylyi*. Thesis. Unpublished observations.

Manchanda V, Sanchaita S, Singh NP. 2010. Multidrug resistant *Acinetobacter*. J. Glob. Infect. Dis. **2**(3):291-304.

Maragakis LL, Perl TM. 2008. *Acinetobacter baumannii*: epidemiology, antimicrobial resistance, and treatment options. Clin. Infect. Dis. **46**(8):1254-1263.

Mattick JS. 2002. The type IV pili and twitching motility. Annu. Rev. Microbiol. **56**:389-314.

Mell JC, Redfield RJ. 2014. Natural competence and the evolution of DNA uptake specificity. J. Bacteriol. **196**(8):1471-1483.

Metzgar D, Bacher JM, Pezo V, Reader J, Döring V, Schimmel P, Marliére P, de Crécy-Lagard. 2004. *Acinetobacter* sp. ADP1: an ideal model organism for genetic analysis and genome engineering. Nucl. Acids Res. **32**(19):5780-5790.

Mortensen BL, Skaar EP. 2013. The contribution of nutrient metal acquisition and metabolism to *Acinetobacter baumannii* survival within the host. Front Cell Infect Microbiol. **3**:95.

Palmen R, Vosman B, Buijsman P, Breek CK, Hellingwerf KJ. 1993. Physiological characterization of natural transformation in *Acinetobacter calcoaceticus*. J. Gen. Microbiol. **139**(2):295-305.

Palmen, R. 1994. Physiological regulation of competence induction for natural transformation in *Acinetobacter calcoaceticus.* Arch. Microbiol. **162**:344-351.

Palmen R, Hellingwerf K. 1997. Uptake and processing of DNA by *Acinetobacter calcoaceticus*—a review. Gene. **192**(1):179-190.

Pontiroli A, Rizzi A, Simonet P, Daffonchio D, Vogel TM, Monier JM. 2009. Visual evidence of horizontal gene transfer between plants and bacteria in the phytosphere of transplastomic tobacco. Appl. Environ. Microbiol. **75**(10):3314-3322.

Porstendörfer D, Drotschmann U, Averhoff B. 1997. A novel competence gene, *comP*, is essential for natural transformation of *Acinetobacter* sp. strain BD413. Appl. Environ. Microbiol. **63**(11):4150-4157.

Porstendörfer D, Gohl O, Mayer F, Averhoff B. 2000. ComP, a pilin-like protein essential for natural competence in Acinetobacter sp. Strain BD413: regulation, modification, and cellular localization. J. Bacteriol. **182**(13):3673-3680.

Salyers A, Whitt D. 2001. Microbiology: Diversity, Disease, and the Environment. Textbook. Fitzgerald Science Press Inc.

Schaik EJ, Giltner, CL, Audette GF, Keizer DW, Bautista DL, Slupsky CM, Sykes BD, Irvin RT. 2005. DNA binding: a novel function of *Pseudomonas aeruginosa* type IV pili. J. Bacteriol. **187**(4):1455-1464.

Seitz P, Blokesch M. 2013. Cues and regulatory pathways involved in natural competence and transformation in pathogenic and environmental Gram-negative bacteria. FEMS Microbiol. Re. **37**:337-363.

Slager K, Kjos M, Attaiech L, Veening JW. 2014. Antibiotic-induced replication stress triggers bacterial competence by increasing gene dosage near the origin. Cell. **157**(2):395-406.

Tapsall JW. 2005. Antibiotic resistance in *Neisseria gonorrhoeae*. Clin. Infect. Dis. **41**(4):S263-S268.

Thomas CM, Nielsen KM. 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat. Rev. Microbial. **3**(9):711-721.

Tu AHT. 2008. Transformation of *Escherichia coli* made competence by calcium chloride protocol. http://www.microbelibrary.org/library/laboratory-test/3152-transformation-of-escherichia-coli-made-competent-by-calcium-chloride-protocol.

Turner L, Stern AS, Berg HC. 2012. Growth of flagellar filaments of *Escherichia coli* is independent of filament length. J. Bacteriol. **194**(10):2437-2442.

Vaneechoutte M, Young DM, Ornston LN, De Baere T, Nemec A, Van Der Reijden T, Carr E, Tjernberg I, Dijkshoorn L. 2006. Naturally transformable *Acinetobacter* sp. strain ADP1 belongs to the newly described species *Acinetobacter baylyi*. Appl. Environ. Microbio. **72**(1):932-936.

Young DM, Parke D, Ornston LN. 2005 Opportunities for genetic investigation afforded by *Acinetobacter baylyi*, a nutritionally versatile bacterial species that is highly competent for natural transformation. Annu. Rev. Microbiol. **59**:519-551.

Zimmer C. 2009. Microcosm: *E. coli* and the New Science of Life. Book. Knof Doubleday Publishing Group.

Figures and Tables.

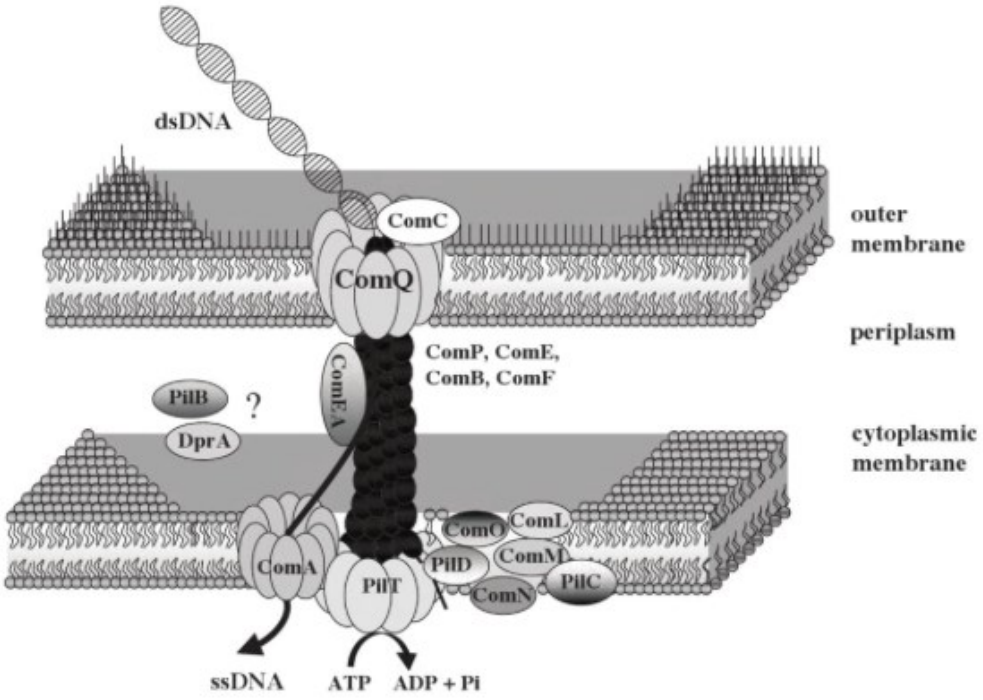


Figure 1. Published hypothetical structure of the competence machine in *Acinetobacter baylyi*

In this study, we investigated genes *comP*, *pilC*, *comA*, *comEA*, *comF*, *pilV*, *pilF*, *fimT*, *comM*, *pilU*, *pilT*, *comC, comE*, *CHP*, *pilG*, *pilR*, and *pilS*. The latter five were deemed not wholly necessary for competence to occur. *comB*, *comF*, *comN*, *comQ*, *pilC*, and *pilD* were tested by Lieber (2014). Remaining ADP1 proteins are either yet to-be-tested for competence by the Lostroh laboratory and/or were deemed essential by de Berardinis *et al* (2008).

Averhoff and Graf (2008) suggested this hypothetical model for DNA uptake in *A. baylyi* ADP1. DNA is bound to protein ComC or the ring of secretin-like ComQ proteins. Upon binding DNA to ComEA, the DNA is transported through the periplasmic space and peptidoglycan mediated by a DNA translocator of pilin-like proteins ComP, ComE, ComF, and ComB. The retraction of the DNA transporter or a ComEA protein traversing the periplasmic space might mediate this step of DNA translocation. A conserved traffic NTPase (PilT) may be essential for the dynamics of the DNA translocator such as pulling DNA through the periplasm via retraction of the DNA translocator. Final translocation across the cytoplasmic membrane is performed through a ComA channel.

Most notably, the difference between Averhoff and Graf's proposed structure and that of the Lostroh laboratory is the distinct lack of a branching external pilus comprised of pilin proteins.

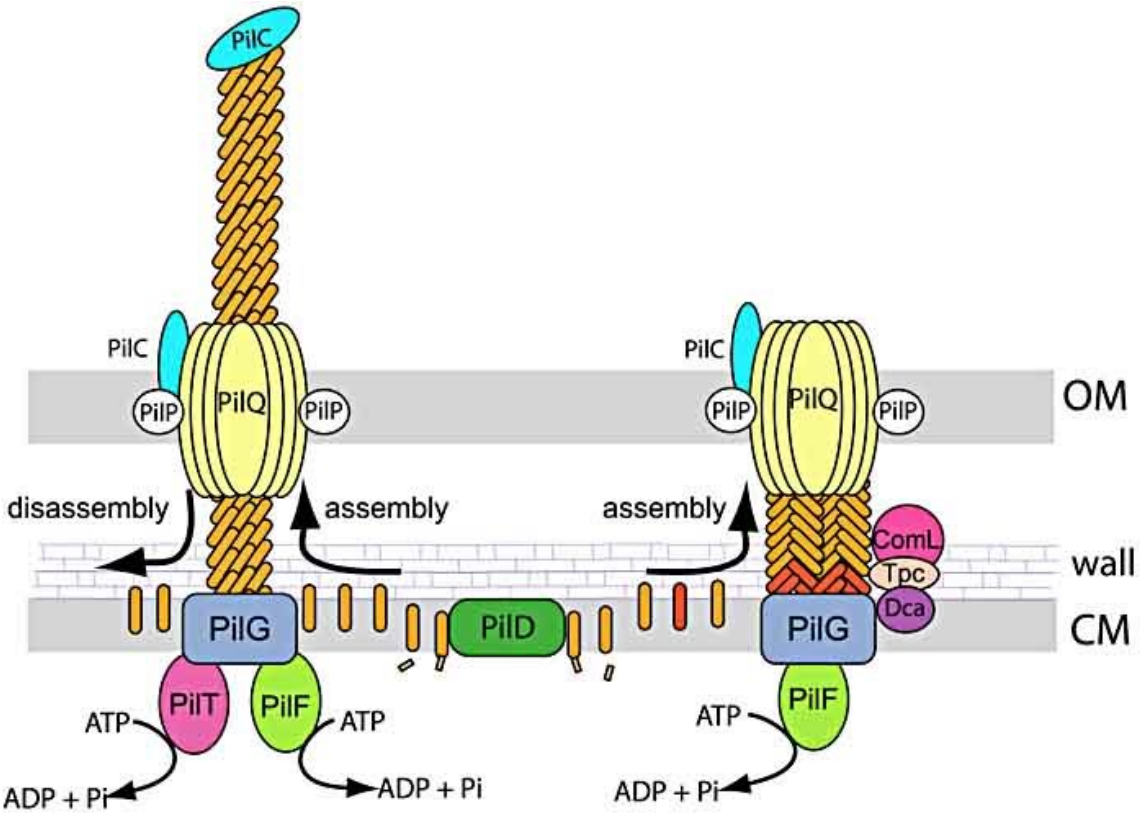


Figure 2. Model for biogenesis of Type IV pilus and DNA uptake and transport machinery in *Neisseria gonorrhoeae*

Like *A. baylyi*, *Neisseria gonorrhoeae* are naturally competent and Gram-negative. In *N. gonorrhoeae*, T4P are involved in both motility and DNA uptake. Chen and Dubnau (2004) designed a hypothetical model for a T4P in *N. gonorrhoeae*. Gram-negative cells have a multi-layered envelope with an inner cytoplasmic membrane (CM), peptidoglycan or wall, outer membrane (OM), and periplasm (between the CM and OM). All protein components in all compartments are synthesized in the cytoplasm and are translocated to respective region.

The "motor subcomplex" is comprised of the inner membrane assembly platform protein, PilC, the motor proteins, PilF and PilT, and the prepilin peptidase, PilD. PilC serves as a base for pilus assembly to begin, PilF utilizes ATP to assemble and extrude the pilins, and PilT utilizes ATM to disassemble and retract the pilus. All pilins start as prepilins, which must be cleaved by PilD before assembly.

In *A. baylyi*, any of the protein components of their homologs could be used for T4P twitching, competence, or both. Some laboratories suggest that competence involves synthesizing a transient pilus, one that is only present during the import of DNA – a "pseudopilus" or even that the pilus structure is never found outside of the periplasm. The Lang and Lostroh laboratory's findings suggest otherwise.

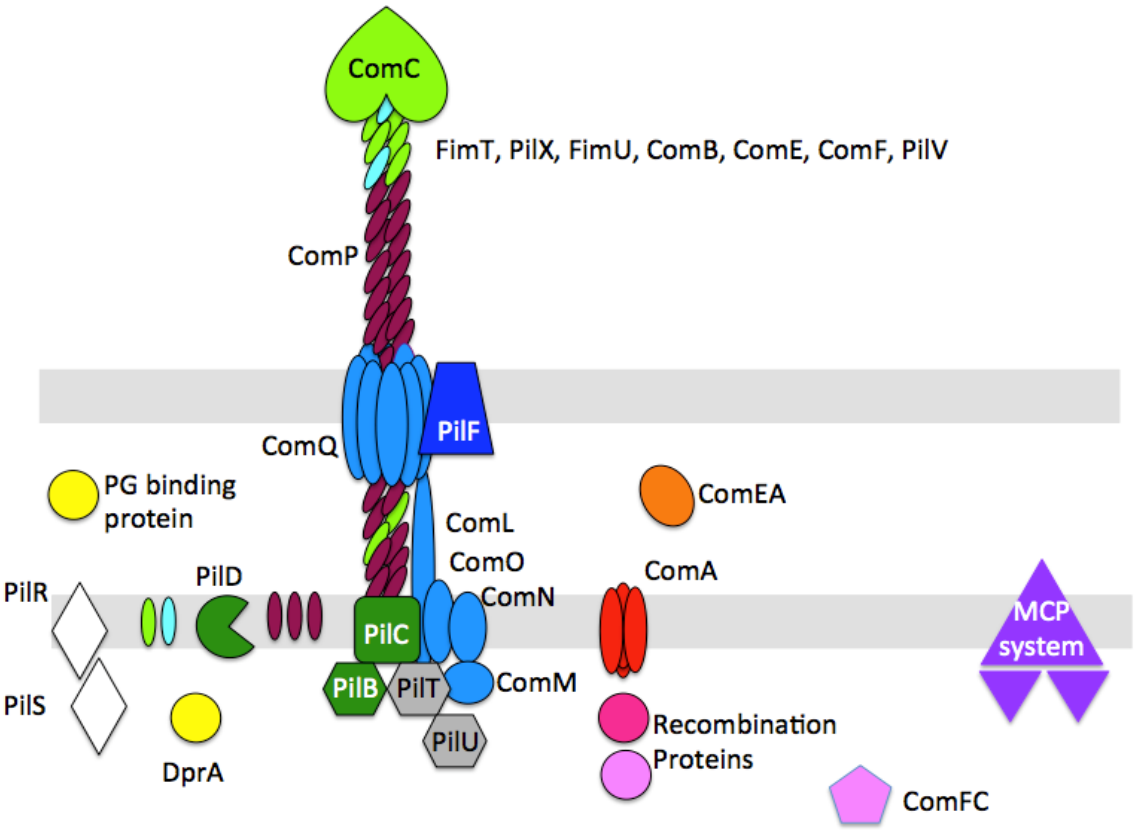


Figure 3. Hypothetical ADP1 competence machine proposed by Lostroh *et al*

The Lostroh *et al*. laboratory designed a hypothetical ADP1 competence machine based on coordinated research performed on knockout mutants in both competence and twitching. Modeled after the *N. gonorrhoeae* model by Chen and Dubnau (2004), Lostroh *et al*. created a mock-up of the competence machinery that notably depicts ComM, PilF, PilC, PilU, and PilT as the main components of the basal apparatus, ComA as an inner membrane DNA translocation protein, ComC as the pilus tip protein, and ComEA as a periplasmic protein thought to help target DNA to the ComA channel. Minor pilin proteins including PilV, FimT, ComF, and ComE and ComP comprise the external appendage.

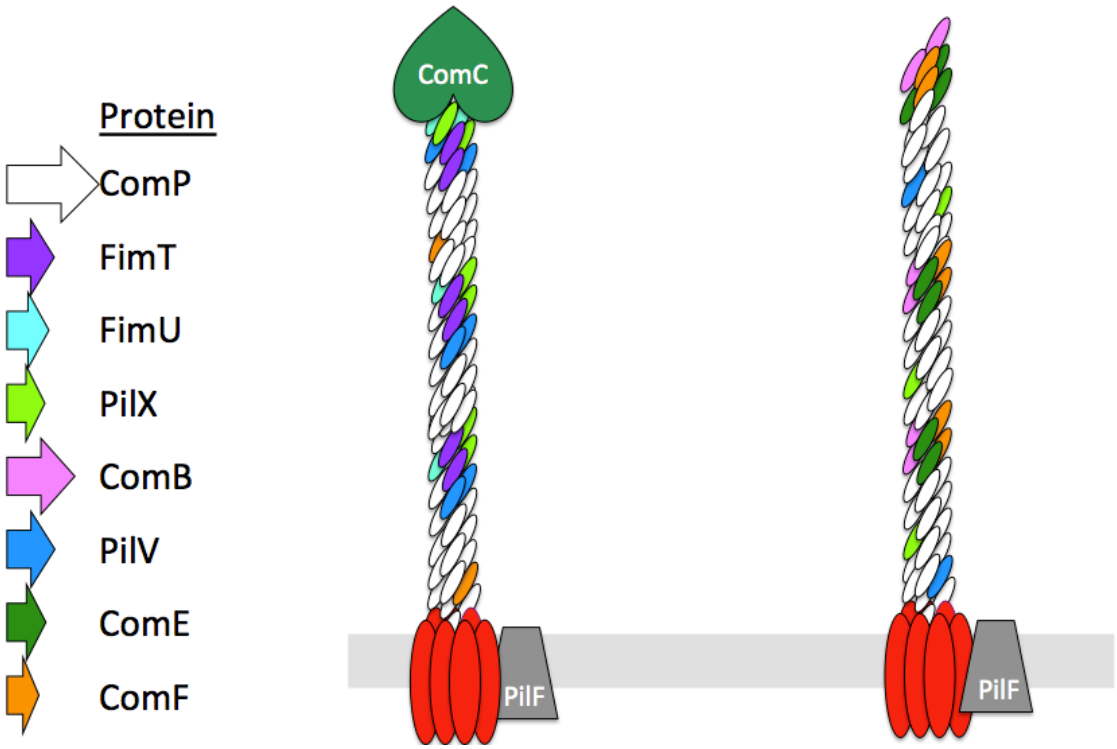


Figure 4. Competence and twitching pili proposed by Lostroh *et al*

In concurrence with Figure 3, the Lostroh *et al*. laboratory designed hypothetical competence and twitching pili based on coordinated research investigating the proteins necessary for each physiological process. ComP (white) comprises the major pilin protein in both the competence (left) and twitching (right) appendages. The basal apparatus of both pili are comprised of a combination of ComM, PilF, PilC, PilU, and PilT. Notably, the twitching appendage lacks the green heart-shaped pilus-tip protein, ComC, which is thought to play a role in DNA binding.

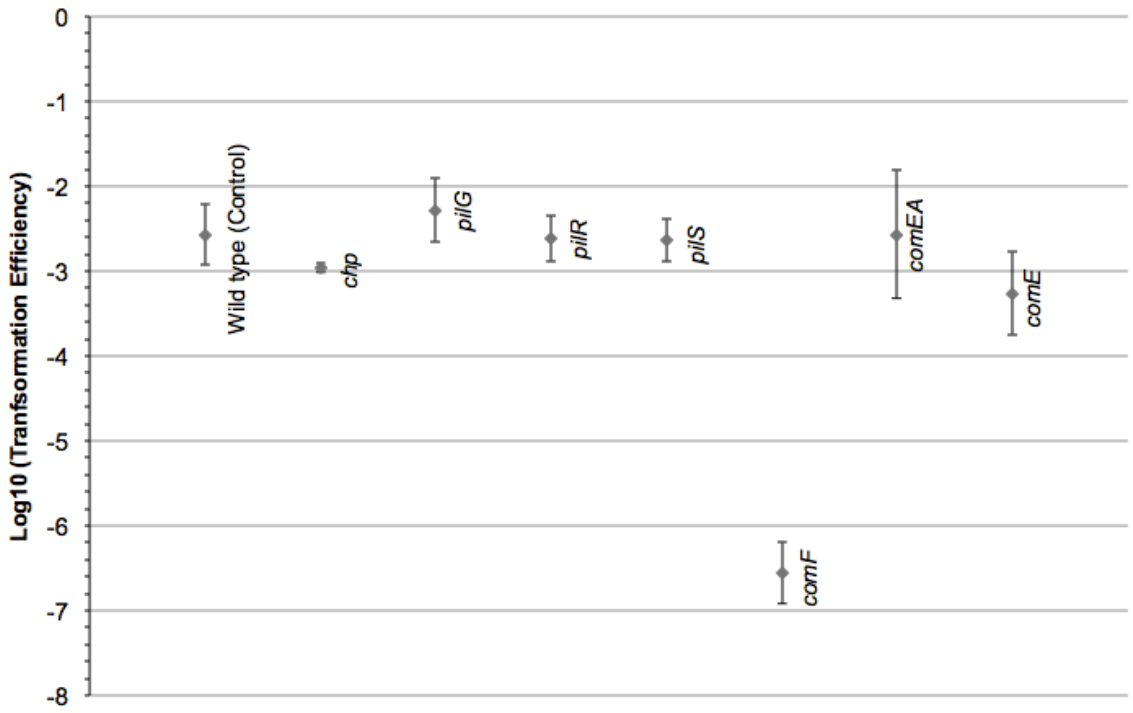
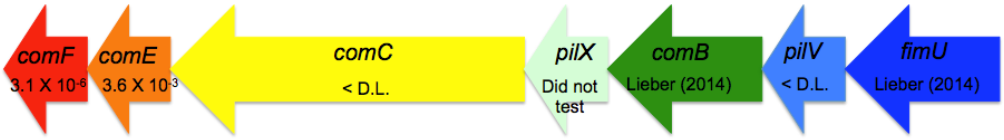


Figure 5. Detectable transformation efficiencies across competent mutants

All of the data in this figure were taken under identical conditions with exception of the specific variation in condition or temperature being tested. Errors bars denote standard deviations, which for this log plot is given by $\Delta z = 0.434\frac{\Delta TE}{<TE>}$ where z = log <TE> and ΔTE is defined as the standard deviation from the mean and <TE> is the mean transformation efficiency.

The data point for the control data contains 15 individual measurements of transformation while the data points for the individual knockout mutants each contain 3 individual measurements of natural transformation. All of these knockout mutants had transformation efficiencies above the limit of detection, suggesting that they are not crucial to the physiological process of competence.



Competence	No	No	Yes	Unknown	No*	Yes	No
Twitching	Unknown	Yes	No	Unknown	Yes	Somewhat	Weakly

Figure 6. Operon containing genes *comF*, *comE*, *comC*, *pilX*, *comB*, *pilV*, and *fimU* and respective transformation efficiency

Colored arrows indicate relative size and directionality of the genes in the operon. Further, the transformation efficiency of each tdk-kan knockout, if applicable, is listed below the gene name. With coordinated efforts by both Lieber (2014) and Rebecca Bloomfield, we found that *comF* is not required for competence but it is unknown if required for twitching, *comE* is not required for competence but required for twitching, *comC* is required for competence but not for twitching, *pilX* has not been tested for competence or twitching, *comB* is required for competence and twitching, *pilV* is required for competence and somewhat required for twitching, and *fimU* is not required for competence and weakly required for twitching.

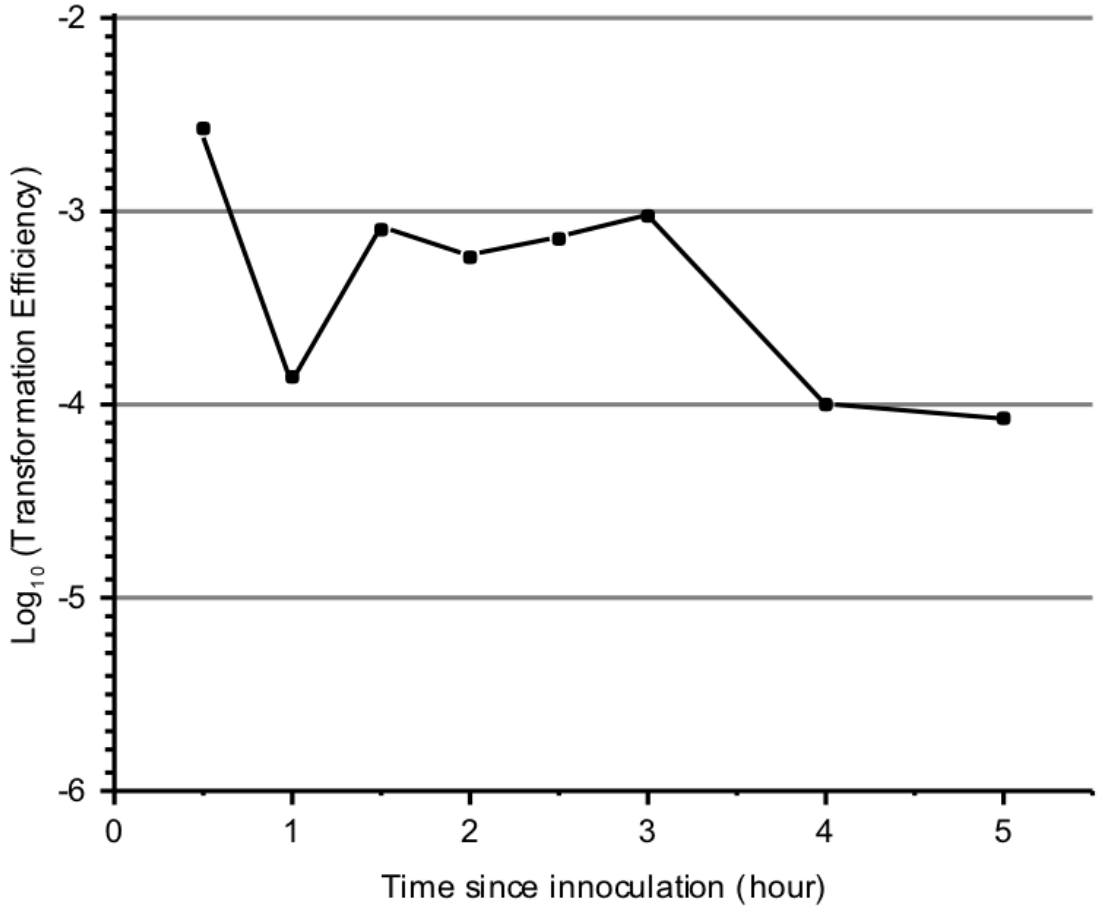


Figure 7. Competence relative to time

This is a summary of three separate transformation efficiency over time experiments. In spite of modifying protocol by varying amounts of DNase added to samples during different experiments, ADP1 competence is high* at 3 hours, indicating the ideal time in which we may collect cells to test for competence. 3 hours serves as a control sampling time. This experiment was performed by Caroline Boyd ’17 and Kaleb Roush ’14.

*Though ADP1 competence does not peak at 3 hours post inoculation, it is the time in which competence is highest prior to dropping off. Further, at 3 hours, we may obtain a great enough quantity of cells for ease of experimentation.

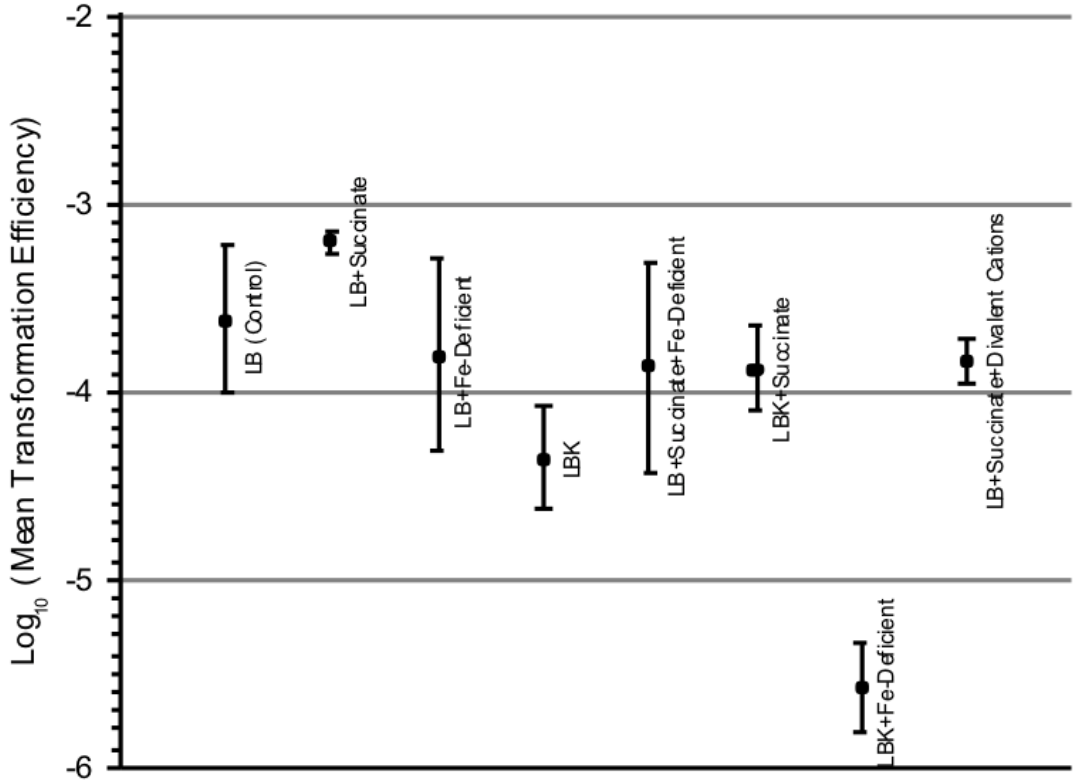


Figure 8. Competence relative to test broth

All of the data in this figure were taken under identical conditions with exception of the specific variation in condition or temperature being tested. We used a single batch of LB and the same incubators throughout the entirety of the test broth experiments and another single batch of LB for the temperature variations. Errors bars denote standard deviations, which for this log plot is given by $\Delta z = 0.434\frac{\Delta TE}{<TE>}$ where z = log <TE> and ΔTE is defined as the standard deviation from the mean and <TE> is the mean transformation efficiency.

The data point for the control data contains 16 individual measurements of transformation while the data points for the variable conditions each contain four individual measurements of natural transformation. LB+succinate yields a transformation efficiency higher than that of control, but LBK+Fe-deficient bears a transformation efficiency considerably lower than that of control.

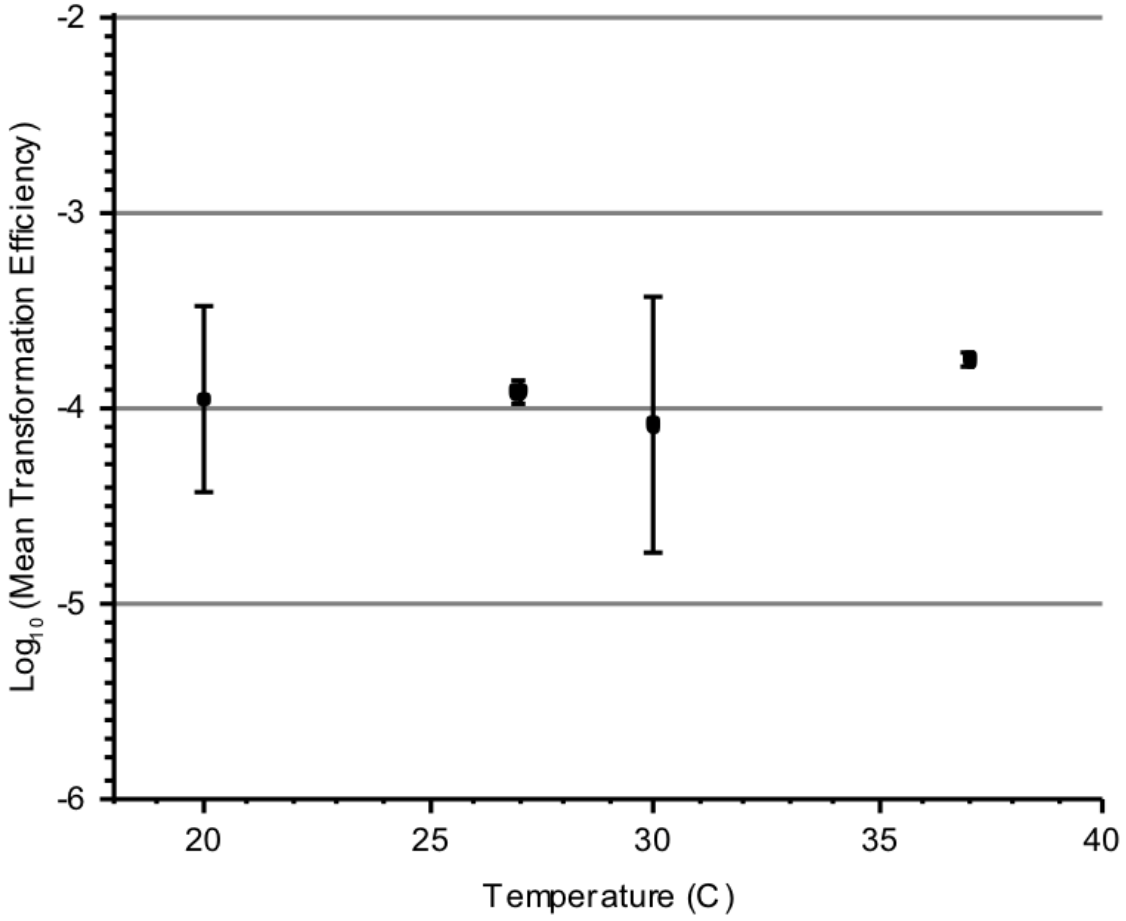


Figure 9. Competence relative to temperature of LB+succinate

All of the data in this figure were taken under identical conditions with exception of the specific variation in condition or temperature being tested. We used a single batch of LB and the same incubators throughout the entirety of the test broth experiments and another single batch of LB for the temperature variations. Errors bars denote standard deviations, which for this log plot is given by $\Delta z = 0.434\frac{\Delta TE}{<TE>}$ where z = log <TE> and ΔTE is defined as the standard deviation from the mean and <TE> is the mean transformation efficiency.

Each data point, including the control, contains 4 individual measurements of transformation. Overall transformation rates do not vary much with temperature, but *A. baylyi* is slightly more competent at 37C than lower temperatures.

Table 1. List of *A. baylyi* mutants used in this study

ACIAD number	Protein missing from mutant	Protein Function*
3338	ComP	Major Pilin
361	PilC	Inner membrane assembly platform required for pilus assembly*
2639	ComA	Channel crossing inner membrane that allows single-stranded DNA to enter cytoplasm*
3064	ComEA	Periplasmic protein that binds to DNA and is required for DNA to enter the cytoplasm through the ComA channel*
3314	ComF	Minor Pilin
3315	ComE	Minor Pilin
3319	PilV	Minor Pilin*
558	PilF	ATPase that associates with PilC and polymerizes pilins to extrude them to build the pilus*
695	FimT	Minor Pilin*
3360	ComM	PilMNOP complex needed for efficient pilus assembly*
3352	CHP	Conserved hypothetical protein
786	PilG	Signal transduction response and regulatory receiver
258	PilR	Signal transduction response and regulatory receiver
259	PilS	Signal transduction response and regulatory receiver for histidine kinase*
911	PilU	NTPase that retracts and disassembles the pilus; potential redundant function with PilT*
912	PilT	NTPase that retracts and disassembles the pilus; potential redundant function with PilU*
3316	ComC	Adhesin found associated to the pilus tip and to the outer membrane; thought to stabilize the pilus fiber*

Appendices.

Appendix 1: Recipes

LB broth:

Using a stir bar, dissolve in 800 mL of tap water: 10 g tryptone, 10 g NaCl, 5 g yeast extract. Pour into 1 L graduated cylinder and fill to 1 L exactly. Pour back into 1 L bottles or flasks; autoclave within an hour.

LB agar:

Using a graduated cylinder, pour exactly 500 mL of LB broth into a 1 L bottle. Add 7.5 g of BD Bacto™ Agar and autoclave within an hour. Add antibiotics, if necessary, immediately before pouring plates, when the agar has cooled to ~55C in a water bath. Avoid creating bubbles, but swirl very thoroughly before pouring to make sure that the agar is distributed equally throughout the solution.

Sterile saline:

Salt solution for diluting cells that are growing in LB and to be plated on LB: 10 g NaCl/L. Dissolve 10 g NaCl in 800 mL of tap water. Pour into 1 L graduated cylinder and fill to 1 L exactly. Cover with parafilm and invert to mix well. Pour into five 250 mL bottles and autoclave within an hour.

***Acinetobacter* minimal medium:**

Component solutions:

To make **0.5 M KH_2PO_4**, dissolve in 80% of the volume of water using formula weight; pour into graduated cylinder and fill to the desired volume. Autoclave and store at room temperature.

To make **10% $(NH_4)_2SO_4$**, dissolve 10 g in 80 mL of water and pour into graduated cylinder and fill to 100 mL. Autoclave and store at room temperature.

To make Hutner's "**Metals 44**," gently warm and dissolve in the following order into 800 mL of dH_2O: 2.5 g EDTA free acid, 10.95 g $ZnSO_4$-$7H_2O$, 1.54 g $MnSO_4$-H_2O, 0.392 g $CuSO_4$-$5H_2O$, 0.250 g $C(NO_3)_2$-$6H_2O$, 0.177 g $Na_2B_4O_7$-$10H_2O$, 100 µL H_2SO_4. Pour into graduated cylinder and fill to 1 L with dH_2O. Store indefinitely at room temperature.

To make **concentrated base**, dissolve 20 g nitrolotriacetic acid in 600 mL of dH_2O. Add 14.6 g of KOH. To this solution, add 28.9 g anhydrous $MgSO_4$, 6.67 g $CaCl_2$-$2H_2O$, 0.019 g $(NH_4)_6Mo_7O_{24}$-$4H_2O$, 0.198 g $FeSO_4$-$7H_2O$, and 100 mL of "Metals 44." Check pH and adjust to pH 6.8 with up to 100 mL of 1 M KOH in dH_2O. Pour into a graduated cylinder and fill to 1000 mL with dH_2O. Pour into clean bottle and store in refrigerator for up to a year. Do not autoclave.

To make **1 M succinate**, multiple formula weight on Sigma Aldrich stock bottle by 0.2, making 0.2 L. Dissolved succinate in 150 mL of dH_2O and poured into a graduated cylinder. Filled to 200 mL and filter sterilized in a 250 mL flask. Store at room temperature.

Acinetobacter *minimal medium agar:*

Obtain two separate 2000 mL flasks. In the first flask, combine 25 mL 0.5 M KH_2PO_4, 10 mL 10% $(NH_4)_2SO_4$, 1 mL concentrated base, 3.35 g Na_2HPO_4, 464 mL dH_2O. In the second flask, combine 18 g BD Bacto™ Agar with 290 mL of dH_2O. Autoclave both of these flasks. Cool to ~55C in a water bath. Add 10 mL of 1 M

succinate to the first flask. Pour the first flask into the second flask and mix well, avoiding bubbles. Pour into sterile plastic petri dishes.

LBK broth:

Using a stir bar, dissolve in 800 mL of tap water: 10 g tryptone, 17.7 g NaCl, 5 g yeast extract. Pour into 1 L graduated cylinder and fill to 1 L exactly. Pour back into 1 L bottles or flasks; autoclave within an hour.

LBK+succinate broth:

After making LBK broth as specified above, add 10 mL 20% succinate (final concentration 0.02% succinate) per 1 L broth.

LBK+Fe-deficient broth:

After making LBK broth as specified above, add 5 mL Santa Cruz Biotechnology 2,2-dipyridyl (final concentration 20 μM 2,2-dipyridyl) per 1 L broth.

LB+succinate broth:

After making LB broth as specified above, add 10 mL 20% succinate (final concentration 0.02% succinate) per 1 L broth.

LB+succinate+Fe-deficient broth:

After making LB broth as specified above, add 10 mL 20% succinate (final concentration 0.02% succinate) and 5 mL Santa Cruz Biotechnology 2,2-dipyridyl (final concentration 20 μM 2,2-dipyridyl) per 1 L broth.

LB+succinate+divalent cations broth:

After making LB-succinate broth as specified above, add 2 mL of 1 M $MgSO_4$ and 100 μL of $CaCl_2$ per 1 L broth (final concentration 2 mM $MgSO_4$, and 100 μM $CaCl_2$).

Appendix 2: Laboratory Protocol

Streaking plates from freezer stocks of bacteria:

Obtain an *Acinetobacter* minimal media agar plate if streaking out wild type ADP1 cells and obtain an LB-kan^{10} agar plate if streaking out *ACIADxxxx::kan* mutants. Both wild type and mutants will be in the -70C freezer in Dr. Phoebe Lostroh's laboratory, refer to the strain book for corresponding CCL numbers. Open the freezer and find the labeled box corresponding to the required CCL number and remove the correct cryogenic vial. Immediately place the cryogenic vial on ice and replace the box and close the freezer door. Taking a long-stem skewer, dip it into the cell slurry and remove a small amount, gently dabbing it on the agar plate. Replace the cryogenic vial in the freezer and streak plate using a metal wand, flaming through a Bunsen burner between streaks.

Crude lysate isolation:

Streak out desired *ACIADxxxx::kan* mutants from cryogenically preserved ADP1 cells obtained from the French collection on LB-kan^{10} agar (de Berardinis *et al*, 2008). Incubate overnight at 37C. Parafilm the plates' edges and store the streak plates in the refrigerator, keeping them for no longer than a week.

Inoculate 2 mL of LB-kan^{10} broth in tubes with single, isolated colonies from the respective mutants' streak plate. Incubate overnight at 37C with high aeration. Transfer 1.5 mL of each mutant culture tube into a single, labeled microfuge tube. Pellet cells in a centrifuge on high speed and discard supernatant. Resuspend cells in sterile water and place microfuge tubes in a heat block at 95C for two hours. This will lyse the cells, releasing their genetic content. At the conclusion of this two hours, microfuge tubes may

be kept on ice until transformation. Create a sterility control LB plate, on which plate 5 µL samples of each mutant's cell lysate to incubate overnight at 37C, assessing sterility.

Moving *ACIADxxxx::kan mutations* into the wild type background:

The same time you streak out desired *ACIADxxxx::kan* mutants, streak out fresh wild type ADP1 *A. baylyi* cells on *Acinetobacter* minimal succinate agar to obtain competent wild type cells. Incubate overnight at 37C. Parafilm the plate's edges and store the streak plates in the refrigerator, keeping them for no longer than a week.

Inoculate 2 mL of LB broth in tubes with single, isolated colonies from the wild type plate. Incubate overnight at 37C with high aeration.

Obtain an LB plate for each mutant to be moved into a fresh wild type background and label accordingly. For each mutant, plate 50 µL of the wild type overnight culture in the middle of the plate. Add 10 µL of lysate from the respective *ACIADxxxx::kan* mutant to the "puddle." Allow the "puddle" to soak into the LB agar and dry (it helps if you perform this step in a hood with a Bunsen burner) and incubate plates overnight at 37C.

The following day, evaluate the sterility control LB plate for growth or lack thereof of the mutant cell lysates. Scrape off the cell "puddle" and transfer sample to 750 µL of sterile saline (10 g/L NaCl), pipetting and vortexing to mix thoroughly. Perform a ten-fold dilution series from 10^0-10^7 in a microtiter plate, diluting with saline (see **Performing ten-fold dilution series**). Plate the ten-fold dilution series on LB-kan^{10} agar, incubating overnight at 37C (see **Plating ten-fold dilution series**). The next day, select dilutions with well-isolated single colonies and pick and streak on LB-kan^{10} agar in duplicate, incubating overnight at 37C. Again, select single colonies from each mutant

streak plate and its duplicate, re-streaking on a new, final LB-kan^{10} plate, incubating overnight at 37C.

Prepare two tubes of -70C frozen glycerol stocks of each new mutant strain from the most recent streak plates. Place 1.5 mL of filter-sterilized 25% glycerol LB in a cryogenic vial. Using a long-stem Q-tip, scrape off all of the cell colonies from a mutant streak plate and inoculate each cryogenic vial. Prepare one cryogenic vial for each mutant plus one additional vial as a duplicate. Store the cryogenic vials at -70C and record new strain numbers per the Lostroh laboratory system in the laboratory strain book.

Crude str^{R} DNA isolation:

This DNA isolation creates a DNA stock to use for many experiments. Streak out cryogenically preserved CCL1011 str^{R} cells on LB-str^{20} agar plates and incubate overnight at 37C. The following day, inoculate six 2 mL tubes of LB-str^{20} with single, isolated colonies from the streak plate of CCL1011. Incubate overnight at 37C with high aeration. Pellet 12 mL of CCL1011 str^{R} cells in eight microfuge tubes. Discard supernatant. Resuspend pellets in a total of 1 mL of sterile LB salts (sterile saline; 10 g/L NaCl). To do this, resuspend pellet in first tube with 1 mL, then transfer all liquid to second tube to resuspend pellet, etc.

Split liquid into two microfuge tubes and add 500 µL of phenol-chloroform-isoamyl alcohol (25:24:1) to each tube. Close tubes and mix by inverting. The chloroform and phenol denatures all proteins and when combined with the isoamyl alcohol, pulls proteins into organic phrase. DNA is left in the aqueous phase. Microfuge tubes for one minute on maximum speed, helping to separate the organic and aqueous layers.

Remove ~450 µL of aqueous solution (containing the DNA) PLUS the interface between the organic and aqueous phase from each tube and transfer to a new microfuge tube. Add 50 µL of 3 M sodium acetate (CH_3COONa) into each tube to make the DNA less soluble in ethanol. Add 1000 µL of 200-proof ethanol (CH_3CH_2OH) to each tube to precipitate out DNA and nucleic acids. Cortex hard and incubate tubes for one hour at -70C to precipitate DNA.

Pellet DNA and salt by spinning tubes in centrifuge on maximum rpm for one minute. Discard supernatant. Open lids of microfuge tubes and allow remaining supernatant to evaporate in 37C incubator. Resuspend each pellet in each microfuge tube in 100 µL of EB buffer (10 mM Tris-Cl of pH 8.5) from a Qiagen DNeasy kit. Combine the two tubes. Obtain LB plate and plate 5 µL of crude str^R DNA to test for sterility; incubate plate overnight at 37C. Store DNA at 4C in the refrigerator. Repeat procedure to create stock of str^R DNA for entirety of experiment. Combine all tubes, invert to mix gently, and take Nanodrop reading ("blank" the machine with Qiagen EB buffer). Finally, split stock DNA into several microfuge tubes for sterility's sake.

Performing ten-fold dilution series:

Obtain a 96 well microtiter plate and a stock of LB salts (sterile saline; 10 g/L NaCl). Place 90 µL of saline into seven wells in a row. Vortex the undiluted sample of cells and then add 10 µL of undiluted cells (10^0) into the first well, pipet up and down to mix, creating the 10^1 dilution. Next, remove 10 µL of cells from the 10^1 dilution and deposit into the second well, pipet up and down to mix, creating the 10^2 dilution. Repeat this procedure until you reach the 10^7 dilution. Repeat procedure for each mutant.

Plating ten-fold dilution series:

To plate the ten-fold dilution series, obtain an LB plate containing the antibiotic marker you are using (either LB-kan^{10} or LB-str^{20}). Draw "pizza slices" on the bottom, diving the plate into eight equal wedges, labeling each slice 10^0-10^7. Pipet two 10 µL drops from each respective dilution, 10^0-10^7, onto each section of the plate. Allow droplets to soak in and dry, it helps to perform this procedure in a hood with a Bunsen burner. Incubate plates overnight at 37C.

Obtaining a growth curve for *A. baylyi* under varying environmental conditions:

Streak wild type cells on *Acinetobacter* minimal media, incubating overnight at 37C. Parafilm the plate's edges and store the streak plates in the refrigerator, keeping them for no longer than a week. Using well-isolated colonies, inoculate four tubes of 3 mL LB broth, incubating at 37C overnight with high aeration.

The following morning, add 1 mL of overnight broth to 24 mL of LB (1:25 dilution) in a side arm flask. In parallel, add 1 mL of overnight broth to 24 mL of LB in a baffled Erlenmeyer flask. This serves as the control. Then, add 1 mL of overnight broth to 24 mL of test broth (1:25 dilution) in a side arm flask. In parallel, add 1 mL of overnight broth to 24 mL of LB in a baffled Erlenmeyer flask. Repeat this so you have two side arm flasks containing test broth and two baffled Erlenmeyer flasks containing test broths. This serves as your two experimental replicates.

Grow all samples for 2 hours at 37C (or other experimental temperature) with high aeration. At 2 hours, begin taking the OD600 of all samples in the side arm flasks (total of three) every half hour. At 3 hours, take a final OD600 reading of the control broth, sampling time. 3 hours post inoculation is the point in the *A. baylyi* growth cycle in which competence achieves its maximum in LB broth at 37C. However, continue to take

OD600 readings of test broth replicates. Sampling time for the test broth is defined as the point in which the OD600 of the test broth is equal to the OD600 of the control sampling time, 3 hours.

At sampling time for both control and test broth, remove 0.5 mL of cells, in duplicate, from the baffled Erlenmeyer flasks and place in a microfuge tube. To each tube, add 5 µL of str^R chromosomal DNA conferring antibiotic resistance. Incubate at 37C with high aeration (to mix DNA) for 1 hour. Add 55 µL of 10X DNase digestion buffer. Add 5 µL of DNase I (at 5 mg/mL in 0.15 M NaCl). Incubate at 37C with high aeration for 15 minutes. Perform a ten-fold dilution series and plate on LB and LB-str^{20} agar to determine transformation efficiency.

Appendix 3: Lostroh Laboratory Strain Book

CCL Number	ACIAD number	Protein missing from mutant	Date frozen down
CCL1780	3338	ComP	01/27/2013 A.D.
CCL2065	258	PilR	07/21/2014 C.L.
CCL2067	259	PilS	07/21/2014 C.L.
CCL2069	361	PilC	07/21/2014 C.L.
CCL2077	558	PilF	07/21/2014 C.L.
CCL2079	695	FimT	07/21/2014 C.L.
CCL2081	786	PilG	07/21/2014 C.L.
CCL2107	2639	ComA	07/21/2014 C.L.
CCL2109	3064	ComEA	07/21/2014 C.L.
CCL2111	3314	ComF	07/21/2014 C.L.
CCL2113	3315	ComE	07/21/2014 C.L.
CCL2115	3319	PilV	07/21/2014 C.L.
CCL2117	3352	CHP	07/21/2014 C.L.
CCL2119	3360	ComM	07/21/2014 C.L.
CCL2131	911	PilU	10/09/2014 C.L.
CCL2133	912	PilT	10/09/2014 C.L.
CCL2135	3316	ComC	10/09/2014 C.L.